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Award Number: DAMD17-97-1-7008

TITLE: Superoxide and Nitric Oxide Mechanisms in Traumatic Brain Injury and Hemorrhagic Hypotension

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REPORT DATE: December 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1999	Final (1 Dec 96 - 30 Nov 99)

4. TITLE AND SUBTITLE
Superoxide and Nitric Oxide Mechanisms in Traumatic Brain Injury and Hemorrhagic Hypotension

5. FUNDING NUMBERS
DAMD17-97-1-7008

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Traumatic brain injury (TBI) renders the brain vulnerable to secondary ischemia and poor outcome after TBI. The goal of this project was to understand the causes of neurological injury due to TBI and hypotension and to use this information to develop treatment strategies to reduce the morbidity of these combined insults. We reported that posttraumatic hypoperfusion can be prevented using L-arginine but that nitric oxide (NO) synthase activity is not affected by TBI, suggesting that TBI is affecting NO directly. We have observed evidence of peroxynitrite (ONOO-) production after TBI and have observed that ONOO- and TBI impair of compensatory cerebral vasodilatory responses. We have performed survival studies after TBI and hemorrhage demonstrating neuronal ischemic injury in 100% of rats subjected to combined injury. Exciting new data suggests that perivascular nerve fibers may modulate myogenic responses through the vasodilatory cannabanoid (CB) receptors. We observed that the destruction of perivascular sensory nerves or CB receptor antagonism reduces myogenic responses to decreased pressure in isolated middle cerebral arteries suggesting that perivascular nerves mediate autoregulation by releasing an agent which dilates the cerebral circulation through CB1 receptors. We observed that hypertonic arginine improves CBF during resuscitation from hypotension after TBI without increasing ICP. These exciting observations cotribute to a better understanding of the mechanisms of traumatic vascular injury and suggest new treatment stategies that will improve functional recovery from TBI and hypotension.

14. SUBJECT TERMS
Head and Spinal

15. NUMBER OF PAGES
191

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Dong S. Ding 12/8/95

PI - Signature Date

(4) TABLE OF CONTENTS

(5). INTRODUCTION:

Abbreviations are defined in the text with first usage but are also defined below:

ACh	acetylcholine
ADC	apparent diffusion coefficient
AUC	area under the curve
CBF	cerebral blood flow
CBV	cerebral blood volume
CB1	cannabinoid receptor type 1
CGRP	calcitonin gene-related peptide
DWI	diffusion weighted image
FID	free induction decay
FPI	fluid percussion brain injury
ICP	intracranial pressure
LDF	laser Doppler flowmetry
L-NAME	L-nitro arginine methyl ester
MAP	mean arterial blood pressure
MCA	middle cerebral artery
MRI	magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
$\cdot O_2^-$	superoxide anion radical
$ONOO^-$	peroxynitrite
PACE	platinized carbon electrode
PSS	physiological salt solution
SOD	superoxide dismutase
TBI	traumatic brain injury
TT	transit time

Traumatic brain injury (TBI) reduces cerebral blood flow (CBF) and renders the brain vulnerable to secondary ischemia. There is clinical evidence that hypotension contributes to poor outcome after TBI, likely because of traumatic damage to the cerebral circulation. Our research has resulted in novel and important observations related to the mechanisms of traumatic vascular injury. We observed that posttraumatic hypoperfusion can be prevented using substrates of nitric oxide synthase (NOS). We also observed that NOS enzyme activity is not affected by our model of TBI, suggesting that TBI is affecting NO directly. We also reported that TBI decreases CBF in rats treated with inhibitors of NOS, suggesting TBI reduces CBF by mechanisms in addition to the destruction of NO. We observed that prolonged (over 45 minutes) superoxide anion radical ($\cdot O_2^-$) production occurs after TBI and that posttraumatic hypoperfusion can be significantly reduced using oxygen radical scavengers. In addition, we reported that while agonist-induced vasodilation and vasoconstriction are intact in rat cerebral arteries after TBI, myogenic responses to hypotension are significantly reduced by trauma. Using magnetic resonance imaging (MRI), we have calculated quantitative, sequential, regional CBF values that are comparable to those reported using other techniques. We have modified techniques for producing progressive, controlled hemorrhage in order to allow us to perform survival studies in rats after TBI and hemorrhage that have demonstrated that TBI and hypotension result in neuronal ischemic injury in 100% of rats subjected to the combined injury. We have observed that L-arginine does not restore myogenic responses after TBI, a result that is consistent with earlier studies demonstrating that CBF autoregulation is not an NO-dependent response. We have extended our *in vitro* studies to demonstrate that peroxynitrite ($ONOO^-$), which is produced when superoxide interacts with NO, reduces cerebral vasodilatory responses to reduced transmural pressure. We have exciting new

data suggesting that perivascular nerve fibers may modulate myogenic responses through the vasodilatory cannabinoid receptors. We observed that the cannabinoid 1 (CB1) receptor antagonist SR141716A significantly reduces myogenic responses to decreased transmural pressure in isolated middle cerebral arterial (MCA) segments. We have noted that phenol, which destroys perivascular nerve fibers, also reduces myogenic responses, suggesting that perivascular nerves may release an agent such as anandamide, which dilates the cerebral circulation by acting at CB1 receptors. We have completed a dose-response study using an arginine-containing hypertonic solution to improve CBF during resuscitation from hemorrhagic hypotension after TBI. A hypertonic saline solution containing 100 mg/kg of L-arginine most effectively restored CBF after hemorrhage.

These exciting observations, which are described in detail below, are consistent with the overall goal of this project: to determine whether a treatment strategy based on our observations will prevent CBF reductions, brain edema, and histological damage after TBI and hemorrhagic hypotension, as well as to understand the mechanisms that contribute to the efficacy of the proposed treatments.

(6). BODY:

This section will summarize progress made in the funding period as related to each Specific Aim.

Specific Aim 1 *is to address the hypothesis that impairment of cerebrovascular function will result in brain injury after TBI and hemorrhagic hypotension that would not occur after hypotension alone. We will relate changes in CBF with MRI evidence of brain edema in rats with and without TBI and hypotension/resuscitation.*

The first set of experiments (SA 1.1 - Effects of vasoconstriction on cerebral vascular input functions), which describes changes in input functions with pharmacologic manipulations, provided important background information about the effects of cerebral vasoconstriction on the input functions which would be used in the CBF calculations. These experiments are described in Mottet, et al. (1)(appendix).

The second set of experiments (SA 1.2 - Quantitative CBF measurements using MRI) describes the use of the input functions characterized in the SA 1.1 experiments for the calculation of quantitative CBF values.

The third set of experiments (SA 1.3 - Quantitative CBF measurements after TBI and hypotension in rats: MRI studies) describes the effects of TBI, controlled hemorrhagic hypotension, and resuscitation on sequential measurements of CBF using MRI.

The fourth set of experiments (SA 1.4 - Effects of TBI and hypotension on brain water: ADC measurements) describes the effects of TBI and hypotension on regional apparent diffusion coefficient (ADC) values. Restriction of water movement (i.e., reduced ADCs) is an indication of increases in brain edema.

The fifth set of experiments (SA 1.5 - Effects of TBI on cerebral arterial constriction and dilation *in vitro*) tests the hypothesis that impaired cerebrovascular function is due to damage to basic mechanisms of constriction or dilation in ring segments harvested from rat cerebral arteries after TBI. These experiments, which are summarized below, have been published (2) (appendix).

The sixth set of experiments (SA 1.6 - Myogenic responses to reduced intravascular pressure) tests the hypothesis that TBI impaired myogenic vasodilatory responses to reduced intravascular pressure (the *in vitro* equivalent of hypotension) in isolated, pressurized segments of MCAs harvested from rats after TBI. These experiments, described in Mathew et al., (3) (appendix), are summarized below.

SA 1.1 - Effects of cerebral vasoconstriction on cerebral vascular input functions

Quantitation of CBF using intravascular tracers requires knowledge of the input function, since the shape of the intracerebral transit curves are affected by the history of the bolus prior to arriving to the brain (4). We have shown that common pharmacological and physiological

manipulations alter the input function (1), yielding erroneous interpretations of relative CBF unless these input function changes are considered. In that paper, we validated the use of both the venous and arterial residual transit curves as components of the input function; the area under the curve (AUC) of a representative pixel in a cerebral vein was utilized as the denominator in the estimation of the cerebral vascular fraction (1). Transit time (TT) estimation of the input function came from a cerebral artery (1). We showed that this method correctly detected changes in CBF after pharmacologic manipulations but grossly overestimated the actual cerebral vascular fraction and produced inaccurate absolute CBF values. More recent efforts have incorporated the input function differently (1)to yield quantitative CBF values. We report here a new way to incorporate the input function that obviates the need for an arbitrary correction factor, yields reasonable estimates of vascular fractions, and results in CBF values within the range reported by other methods. Specifically, we have included the sum of all pixels identified as a cerebral vein as the venous residue function. Summing these pixels seems to satisfactorily represent the bolus of contrast agent presented to the brain and a more physiologically accurate denominator for the determination of brain vascular fraction.

SA 1.2 - Quantitative CBF measurements using MRI.

For these experiments, rats (n=13) were anesthetized with isoflurane, intubated, and injected intravenously with gadolinium diethylenetriamine pentaacetic acid (DTPA), and bolus transit curves were obtained with a fast gradient echo as reported (1). Body temperature was maintained with a warm water blanket. For the input function, veins and arteries visible on the same slice used for the brain transit curves were located. These regions were hyperintense at baseline and decreased and recovered intensity in temporal sequence corresponding first to the artery, then to the brain, and finally to the vein. The artery was used for TT calculations. The bright region in the baseline image that subsequently darkened with bolus passage representing a vein was outlined by hand, and the area under this entire venous curve used later in the calculation of vascular volume fraction. Mixed gray/white brain regions in both hemispheres were analyzed for the results reported here. Operator-defined arithmetic integration of the transit curves was used instead of fitting the curve to a gamma-variate function because many of the residue curves, particularly those from the veins, appeared to be composed of multiple compartments and recirculation. These curves then were analyzed for mean TT (from the arterial and brain parenchymal curves) and the brain vascular fraction (from the AUC of the venous and parenchymal curves). True TT was obtained by subtracting the TT obtained in the artery from that obtained in the brain, and the vascular volume fraction was obtained by the ratio of the mean AUC in the brain to the AUC under the transit curve of the sum of the venous pixels. The vascular fraction was converted to ml/g, using a previously determined intensity/concentration function (1), and the density of brain vs. blood. CBF was then calculated as the vascular fraction/True TT (multiplied by 60 sec/min), and then expressed per 100 g brain tissue. The resulting units were ml $\cdot$ min^{-1} $\cdot$ 100 g^{-1}.

Because of limited spatial resolution, the arterial curve was sometimes difficult to reliably locate and often included a small tail that likely represented volume-averaging with adjacent brain or vein, but the curve could be identified in most cases. Susceptibility artifacts also blurred the borders of the cerebral veins and the identification method was subsequently modified to include as venous only those pixels that clearly darkened with bolus passage. Despite these difficulties, the model yielded reasonable results (see table):

Baseline CBF (ml $\cdot$ min^{-1} $\cdot$ 100 g^{-1})	Baseline TT (sec)	Baseline CBV (ml/100g)
117 ± 14	0.89 ± 0.10	1.49 ± 0.14

The original tracer kinetic models were applied to other methods, such as radioisotope methods, in which external detection or artificial reference organs readily yield values related to the

total amount of contrast agent presented to the brain. Alternatively, our previous approach (using a representative voxel for the input function), while valid for relative measurements, was not sufficient for quantifying the vascular fraction. By summing the venous voxels, the apparent vascular volume fraction using this method is closer to the actual vascular fraction than using single pixel values, and CBF is within values obtained using radioactive microspheres in rats anesthetized with isoflurane (5,6). This model was used to measure CBF, cerebral blood volume (CBV), and TT during and after TBI and hypotension in the experiments described below.

SA 1.3 - Quantitative CBF measurements after TBI and hypotension in rats: MRI studies

Male Sprague-Dawley rats were surgically prepared for MRI CBF measurements and TBI, as described below, and randomly split into four groups in order to determine the individual and combined effects of mild hypovolemic hypotension and TBI on CBF using the technique of contrast agent bolus-tracking MRI.

Male Sprague-Dawley rats (452±10g, n=26) were anesthetized with 4% isoflurane in O_2:air (50:50), intubated, and mechanically ventilated at an isoflurane concentration of 2% in O_2:air (20:80) at a rate of 20-30 bpm. Two femoral arteries and one femoral vein were cannulated with PE90 tubing containing heparinized plasmalyte (5 U/ml). One arterial line was used from then on for continuous monitoring of mean arterial pressure (MAP). Core temperature was maintained using a circulating water bath/blanket and monitored continuously via a rectal temperature probe. After placement in a stereotactic holder, a midline incision was made in the scalp. The scalp and underlying fascia were retracted for a 5-mm outside diameter craniotomy (midway between the Lambda and Bregma, 3.5 mm to the right of the midline suture). Once the craniotomy had been cleared, a machined trauma adapter made from a 20 gauge needle hub was cemented over the craniotomy using cyanoacrylic and dental acrylic. The adapter was filled with saline and attached to the fluid transmission tube, which allows for continuous transmission of the injury pulse from the fluid percussion injury (FPI) device to the right cortex of the rat. The animal was wrapped in a water blanket for the remainder of the experiment. At this point a blood sample was taken to ensure that hemodynamic parameters were within acceptable limits. MAP was maintained between 95 and 105 mm Hg by adjustment of the isoflurane concentration (typically between 1.4 and 1.0%).

After preparation, the rats were placed in a specially designed plexiglass cradles and each cradle was placed in the magnet (Oxford Instruments, 4.7T). The coil was tuned to the proton frequency (200.056 MHZ), and the magnetic field throughout the sample volume was then maximized by shimming on the water free induction decay (FID). Once a series of pilot images had been acquired to allow for planning of the transverse imaging slices, a high-resolution image was acquired and the FPI device was calibrated and connected to the trauma adapter. Another high-resolution image was then acquired, followed by a diffusion weighted image (DWI) experiment (three diffusion weighting of 0, 3 and 5 gauss/cm, tr = 3 sec, nv = 128, nt = 1) and a high-resolution reference movie (TE = 0.003 sec, tr = 0.008 sec, nv = 128, nt = 2). Rats were randomly assigned to one of the following groups:

Group One = TBI + 45 minutes hemorrhagic (hypovolemic) hypotension at 60 mm Hg
Group Two = TBI
Group Three = Sham injury + 45 minutes hypovolemic hypotension at 60 mm Hg
Group Four = Control (sham injury)

The bolus-tracking movie was then conducted using the same set of acquisition parameters except that nv and nt were reduced to 64 and 1, respectively, thus producing a total acquisition time for each frame of 0.512 sec. A bolus of 0.3 ml contrast agent (OMNISCAN, gadodiamide) was introduced during the sixth frame of this movie and took approximately 1.5 seconds for the total injection through the femoral vein. After each bolus-tracking movie was made, a washout period of 20 minutes was used to reduce build-up of the contrast agent via renal filtration and excretion. After the washout period, MAP was lowered to 60 mm Hg by removal of whole blood from one of the femoral arterial lines. Another reference movie followed by a bolus-tracking movie was then conducted, immediately after which the blood was reinfused to reestablish MAP at 100 mm Hg. A

washout period followed, then a DWI and another reference + bolus-tracking movie pair. Fluid-percussion-induced TBI took place (Groups One and Two) after the washout, followed immediately but another reference + bolus-tracking movie pair. At the end of the washout period, MAP was once again lowered to 60 mm Hg by removal of whole blood and another reference + bolus-tracking movie pair acquired. MAP was maintained at 60 mm Hg for Groups One and Three, while for rats in Groups Two and Four it was restored to 100 mm Hg as above. A DWI was then conducted at the end of the washout and then another reference + bolus-tracking movie pair acquired. For Groups One and Three, MAP was then restored to 100 mm Hg as above. A final DWI and reference + bolus-tracking movie pair were then acquired to determine the results of resuscitation.

K-space filling of the bolus-tracking movie using the reference movie was performed to allow for enhanced spatial resolution. Movies were then constructed in a file format for use in an on-site developed software package (Transit) and analyzed using the following methodology. An artery and vein at the base of the ipsilateral hemisphere of the brain were selected to provide the input and post-cerebral vascular functions, respectively. From this data, the passage time (To-TOA) of the bolus in seconds, as well as the total signal observed, was determined for each vessel. The majority of the parenchyma of the brain was then outlined and the average flow curve was determined to give the AUC and To-TOA. These data were then used to calculate the TT (in seconds), CBV (expressed as percent of total brain volume), and CBF (expressed as ml · 100 g^{-1} tissue · min^{-1}).

The experiment design is diagrammed below (Figure 1):

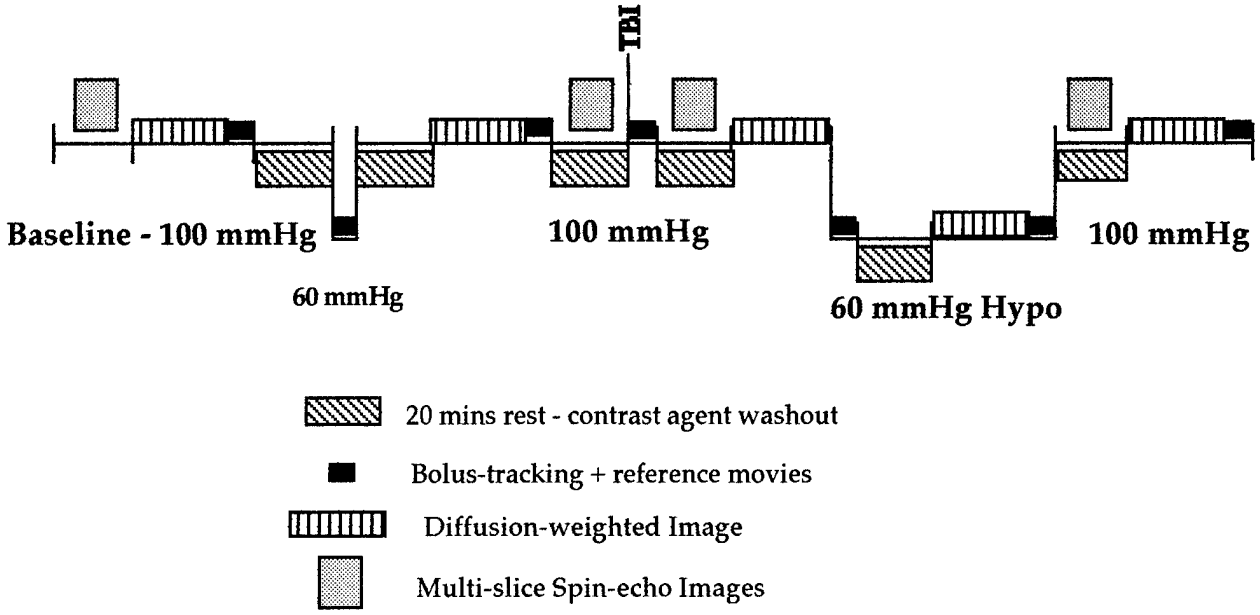


Figure 1: Timeline illustrating the sequence of experiments carried out on each rat in this study. The 20 minute rest periods were included to allow washout of the contrast agent to allow for effective continuation of contrast enhancement during successive bolus-tracking movies. All pressures indicate the target MABP monitored via a femoral arterial cannula.

Physiologic parameters

There were no significant differences were observed between any of the groups with regard to: total Hb, $\%O_2Hb$, $\%CO_2Hb$, %MetHb, $\%volO_2Ct$, Hematocrit, MAP and rectal temperature. However, a significant difference in rat weight was observed between the TBI-only and Hypo-

only groups (unpaired t-test, p = 0.0483).

No significant changes were observed for: pCO_2, pO_2, $\%O_2Hb$, %COHb, %MetHb, MAP, or rectal temperature in all groups when final values were compared to the baseline values presented above. Yet, significant decreases were observed for the following groups: blood pH for TBI+Hypo (p = 0.0402) and Control (p = 0.015), blood HCO_3^- for TBI+Hypo (p = 0.0140), blood TCO_2 for TBI+Hypo (p = 0.0148), total Hb (g/dL) for TBI+Hypo (p = 0.0002), blood %O2 volume drop for TBI+Hypo (p = 0.0002) and Hypo-only (p = 0.0269), and hematocrit for TBI+Hypo (p = 0.0462).

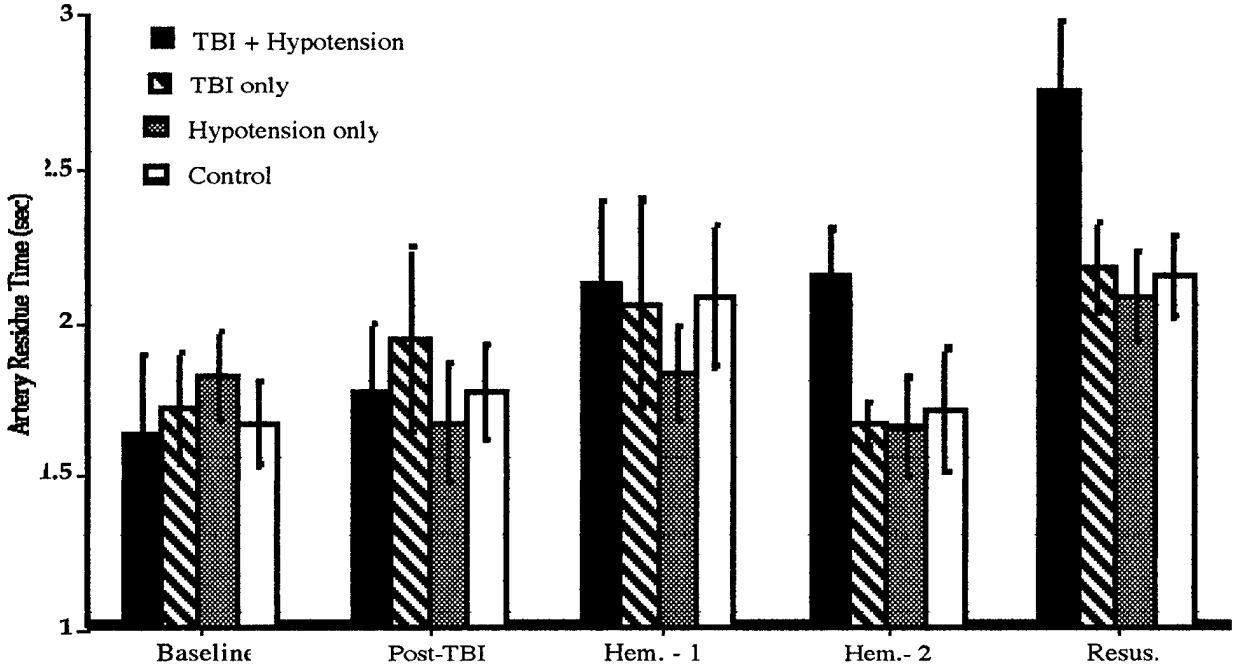


Figure 2: Changes in arterial bolus residue time over the course of the experiment, showing the differences between the groups as well as the variation with time. Note that the Y axis does not begin at zero. BL100 = baseline at MAP of 100 mm Hg, Post-TBI 100 = after TBI at MAP of 100 mm Hg, Post-TBI 60 = after TBI at MAP of 60 mm Hg. Results as shown are average for the group represented by the bars and the error bars show SEM.

Arterial bolus residue parameters:

The arterial residue time for the bolus increased in all groups during the control and post-TBI hypotensive periods (first and last control group bars respectively)(Figure 2).

All four groups exhibited the same behavior until the end of the 45 minutes hypotensive period, where it can be seen that the TBI+Hypo group arterial transit times remained higher than the other three groups (which exhibited the same end value) for the remainder of the experiment.

This difference between the TBI+Hypo group and the other three groups is almost significant (p=0.0767, p=0.0601 & p=0.1068 respectively) at the final point, whereas, at the previous point they were 0.0160, 0.0437, & 0.0855 respectively.

Arterial residue curve AUC (Figure 3) showed a significant increase (p=0.0005) during the baseline autoregulatory challenge and then decreased significantly (p=0.0038) to a value similar to the initial baseline measurement (p=0.3774), determined using paired t-tests.

Calculated Parameters:

	Mean TTT	TTT SEM	Mean CBV%	CBV SEM	Mean CBF	CBF SEM
BL100	0.7567	0.0761	1.72	0.15	176.27	21.00
BL60	1.0246*	0.0838	1.86	0.16	169.74	40.93
BL100	1.0547	0.1186	1.88	0.15	139.52	23.14

Table 1: Calculated parameters for the first three bolus-tracking movies. The asterisk (*) indicates a significant increase ($p < 0.05$) from the first bolus-tracking movie (BL100) to the autoregulatory challenge (BL60).

No differences were observed between any of the groups in calculated TTT over the time-course of the experiment. At the end of the 50 minute hypotensive period the Hypo-only group showed a higher average than the other three groups, but this did not prove to be significant ($p = 0.0796$, data not shown).

It can be seen in the above table that the overall mean TTT appears to have significantly increased from baseline to acute hypotension ($p = 0.0289$), but did not return to the previous value after reinfusion of whole blood to maintain a MAP of 100 mm Hg.

Vascular volume of the brain showed no significant differences between the groups and was essentially unchanged throughout the duration of the experiment for the TBI+Hypo, Hypo-only and Control groups. This was not the case for TBI-only group, which showed an overall decrease during the experiment, resulting in a significantly lower CBV by the end of the experiment ($p = 0.0227$). No change in overall mean CBV was observed during the baseline autoregulatory challenge.

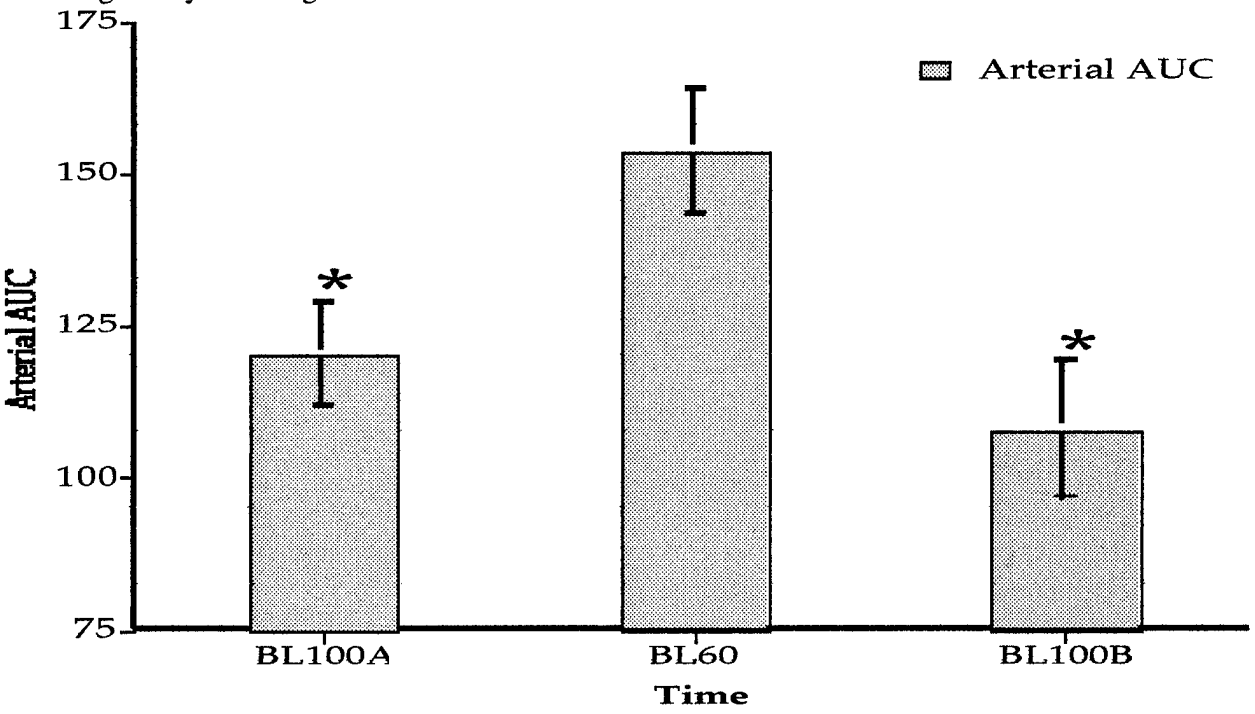


Figure 3: Changes in vein bolus residue time over the course of the experiment with respect to the first measurement (set at 100%). Note that the Y axis does not begin at zero. BL100 = baseline at MAP of 100 mm Hg, Post-TBI 100 = after TBI at MAP of 100 mm Hg, Post-TBI 60 = after TBI at MAP of 60 mm Hg. Results as shown are average for the group represented by the bars and the error bars show SEM.

SA 1.4 - Effects of TBI and hypotension on brain water: ADC measurements.

ADCs were calculated from the experiments described above. DWI is implemented as a modification of the standard spin-echo sequence. Contrast in the diffusion-weighted spin echo image (DWI) results from differences in tissue water diffusion (7). TR is 3.0 s and TE is 60 ms. Two 18-ms rectangular-shaped, diffusion gradient pulses are added symmetrically on either side of the refocusing 180 rf pulse. The diffusion gradient separation time, Δ, is 29 ms. The diffusion gradients are set at 1.0 and 4.0 gauss/cm, which produces attenuation factors (b values) of 1000 and 2000 mm^2/s (7). By plotting the natural log of the intensity vs. the b value for the T2WI image (b value essentially zero) and the two DWI images, we calculated apparent diffusion coefficients in the dorsal (i.e., adjacent to the trauma site) and ventral cerebral hemispheres and basal ganglia and thalamus, both ipsilaterally and contralaterally to the injury site. ADCs were calculated at baseline (Baseline), immediately after TBI (Post-TBI), at the beginning (Hem.-1) and end (Hem.-2) of the 45-minute period of hemorrhagic hypotension and after resuscitation (Resus.).

Repeated measures analysis of variance revealed significant time ($p = 0.0036$) and group ($p = 0.013$) effects as well as a significant time vs. side vs. group interaction ($p < 0.0001$).

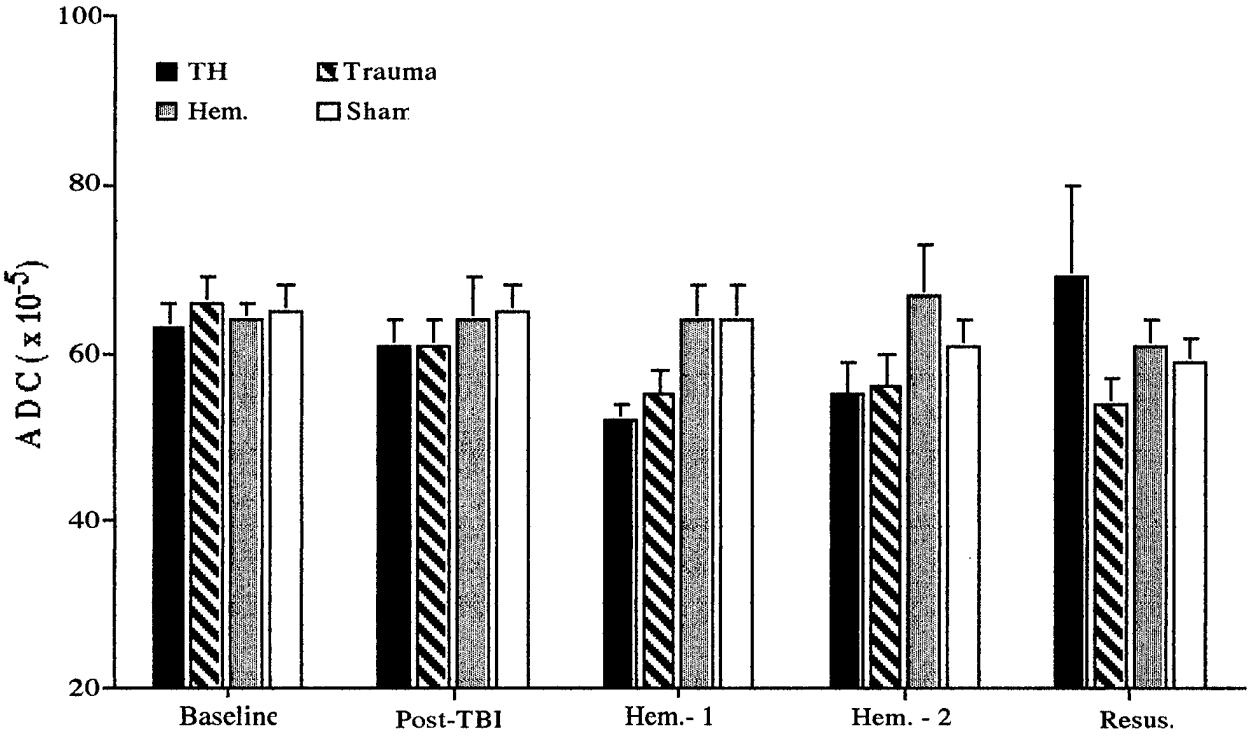


Figure 4 - Apparent diffusion coefficients (ADC, $mm^2/sec \times 10^{-5}$) in the ipsilateral cerebral hemisphere adjacent to the site of fluid percussion TBI. TH = trauma+hemorrhagic hypotension group; Trauma = trauma-only group; Hem. = hemorrhagic hypotension-only group; Sham = sham injury (no trauma, no hypotension).

ADCs in the hemisphere adjacent to the injury site remained stable in the hypotension-only and sham injury groups throughout the measurement interval (Figure 4). Neither of these groups were subjected to TBI and ADCs were similar between the Baseline and Post-TBI measurement periods. ADCs did not change in the hemorrhage-only group during the period of hypotension, indicating that, in the absence of other insults, cerebral edema does not occur during a 45-minute period of moderate hemorrhagic hypotension. In the trauma-only group, ADCs decreased very slightly immediately after TBI but then decreased and remained below baseline for the remainder of the experiment. ADCs decreased in the trauma+hemorrhage group during the period of hemorrhagic hypotension but then increased above baseline following resuscitation.

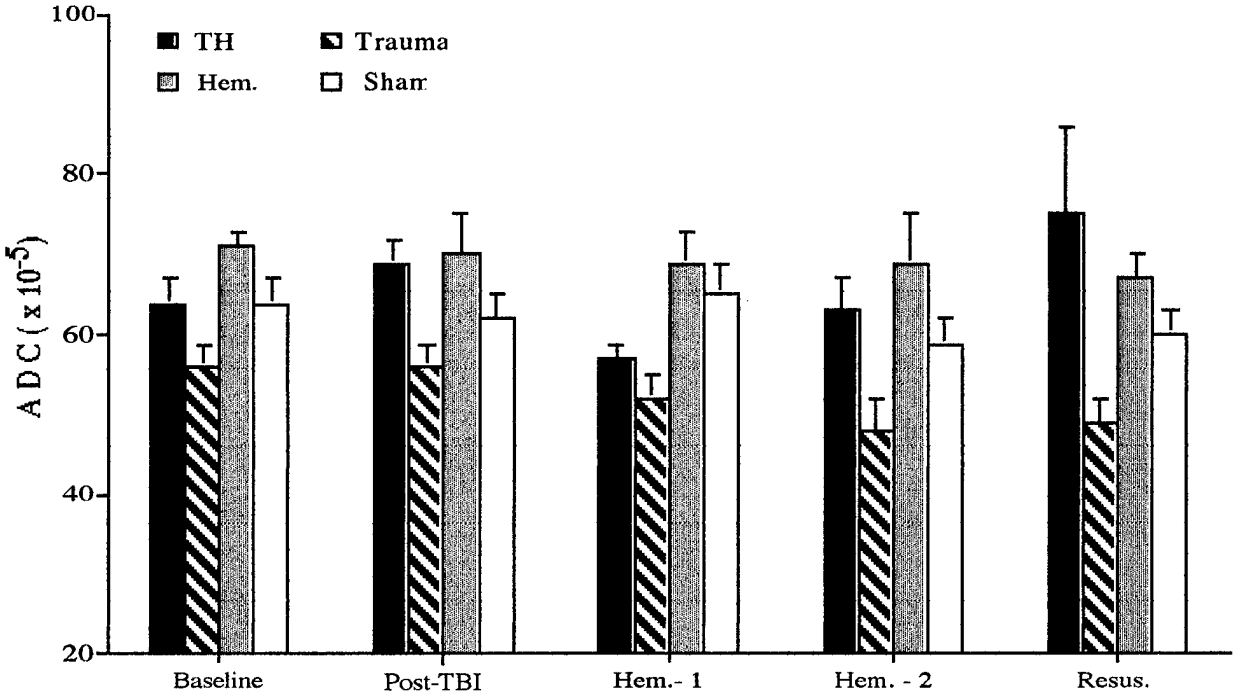


Figure 5 - Apparent diffusion coefficients (ADC, $mm^2/sec \times 10^{-5}$) in the ipsilateral cerebral hemisphere ventral to the site of fluid percussion TBI. TH = trauma+hemorrhagic hypotension group; Trauma = trauma-only group; Hem. = hemorrhagic hypotension-only group; Sham = sham injury (no trauma, no hypotension).

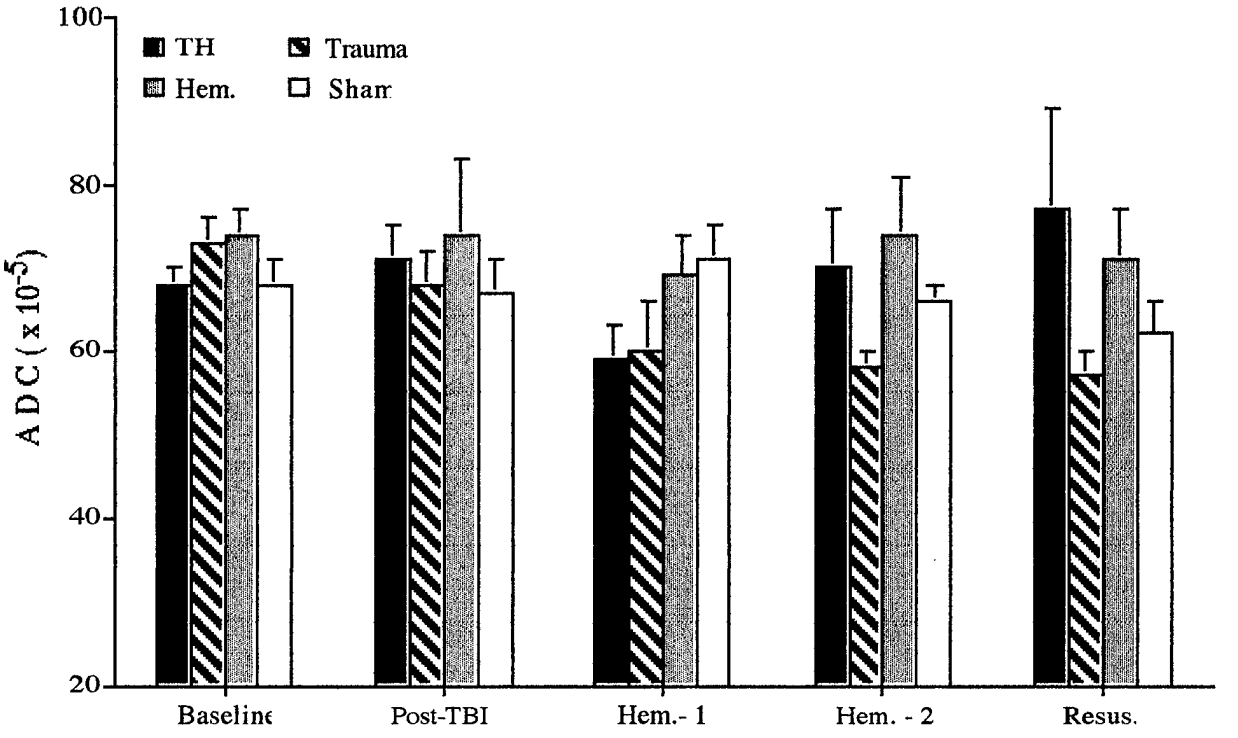


Figure 6 - Apparent diffusion coefficients (ADC, $mm^2/sec \times 10^{-5}$) in the ipsilateral thalamus. TH = trauma+hemorrhagic hypotension group; Trauma = trauma-only group; Hem. = hemorrhagic hypotension-only group; Sham = sham injury (no trauma, no hypotension).

The ipsilateral cerebral hemisphere distant from the injury site (Figure 5) and the ipsilateral thalamus (Figure 6) exhibited similar ADC patterns. ADCs remained stable in the hypotension-only and sham-injury groups. ADCs decreased in the trauma-only group and remained below baseline throughout the experiment. In the trauma+hypotension group, ADCs decreased at the beginning of the period of hemorrhagic hypotension but then returned to baseline by the end of the hypotensive period and then increased above baseline during resuscitation. The results from the sham injury group indicate that ADCs don't change over time, suggesting that the experimental preparation and the aesthetics used don't increase brain edema during the experimental period. The results from the hypotension-only group indicate that ADCs did not change during or after hypotension, suggesting that moderate, transient, hemorrhagic hypotension does not cause edema in the absence of TBI. The results indicating that ADCs decreased after TBI in the trauma-only group suggest that TBI caused increases in brain edema. Since ADCs decreased after TBI in all brain regions on the injured side, our results indicate that brain edema occurs throughout the ipsilateral hemisphere after TBI and that the edema persists for hours after TBI. ADCs decreased after during hypotension in the trauma+hypotension group but, surprisingly, returned to or above baseline ADC values by the end of the hypotensive period and/or during resuscitation. These results suggest that edema occurred after TBI in the trauma+hemorrhage group but that the edema resolved during the hypotensive period in brain regions away from the injury site and during resuscitation in the hemisphere adjacent to the injury site.

SA 1.5 - Effects of TBI on cerebral arterial constriction and dilation *in vitro*

To determine whether TBI altered cerebral vascular responsiveness to vasoconstrictors or vasodilators, rats were anesthetized with isoflurane (1.5-2.0% in air:O_2), prepared for central fluid-percussion TBI, and subjected to moderate injury (2. 3 atm., n=8) or sham injury (n=8), after which, isoflurane concentrations were increased from 1.25% to 2.0% and rats were exsanguinated via cardiac puncture. The brain was excised, and the posterior cerebral arteries were removed and mounted on a wire myograph. Arterial segments were studied intact or with the endothelium denuded by gently rubbing the lumen with a human hair. After an equilibration period of 30 minutes at 37°C, the segment was set to its optimal length for force development by construction of an active length-tension curve using 100 mM K^+ as an agonist. Concentration-response curves were generated using the cumulative addition of serotonin or arginine vasopressin. Vasodilator responses to acetylcholine (ACh) were studied after constriction with 10 μM serotonin. Contractile responses were plotted as active tension vs. log of the molar concentration of agonist, and force responses were expressed in units of mN normalized to axial length (active tension = mN/mm). Force generation to serotonin or ACh was unaffected by TBI (Figure 7 & 8). We also tested cerebral vascular responses after severe (3.0 atm) TBI and in middle cerebral as well as posterior cerebral arteries and found no effect of TBI on vasoconstrictor or vasodilatory responses. To test the hypothesis that traumatic vascular damage would increase with time *in situ*, rats were allowed to survive for 30 minutes after TBI before vessels were harvested. There was no alteration in force generation in these rats. These studies demonstrate that cerebral arterial smooth muscle from middle and posterior cerebral arteries remains capable of normal contraction and dilation after both moderate and severe TBI.

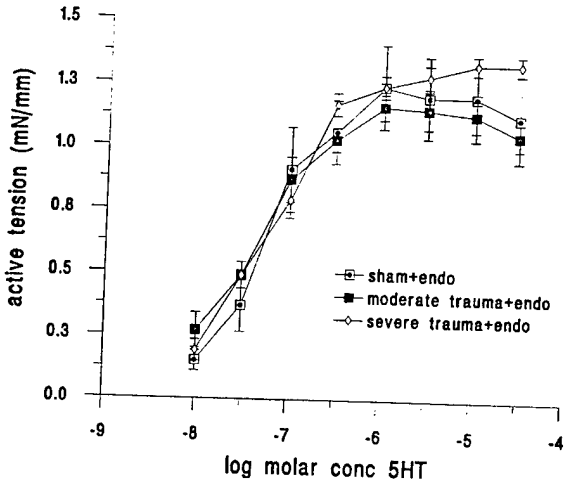


Figure 7. Active tension to serotonin (5HT) in posterior cerebral artery rings from rats after sham-TBI, moderate (2.3 atm) TBI, or severe (3.0) TBI.

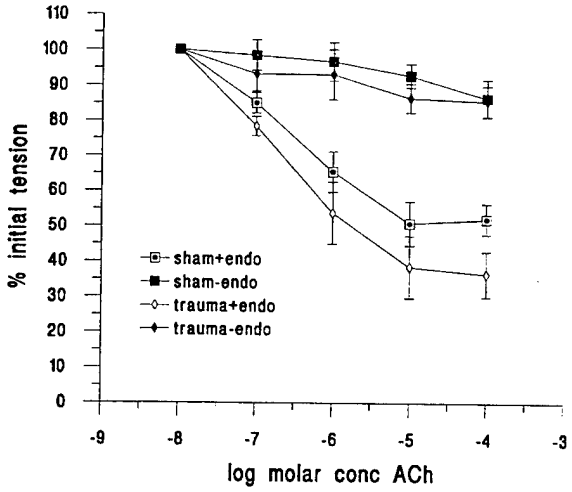


Figure 8. Active tension to acetylcholine (ACh) in posterior cerebral artery rings from rats after sham-TBI or moderate (2.3 atm) TBI expressed as percent of maximum serotonin-induced vasoconstriction.

SA 1.6 - Myogenic responses to reduced intravascular pressure

To determine whether pressurized segments of rodent cerebral arteries would respond normally to decreases in intravascular pressure, rats were anesthetized with isoflurane, prepared for central fluid-percussion TBI and subjected to moderate TBI (2.2 atm) or sham-TBI. The rats were then exsanguinated via cardiac puncture and the brains were excised and sections of the MCAs were removed and mounted and tied onto glass micropipettes in an arteriograph. Arterial segments were bathed in physiological saline bubbled with 20% O_2, 5% CO_2, and 75% N_2 at 37°C. Intravascular pressure was set at 100 mm Hg by raising reservoirs connected to the micropipettes. Arterial diameters were measured through an inverted microscope connected to a video camera and monitor and a video scaler. Measurements were performed by an investigator who was blinded as to the treatment groups. Intravascular pressure was decreased progressively in 20 mm Hg increments, and the change in vessel diameters was measured. Rats were randomly assigned to 1 of 4 groups (sham/5 min harvest; sham/30 min harvest; TBI/5min harvest; TBI/30 min harvest; n=4 per group). In sham-injured rats (Figure 9, sham-5 and sham-30), the MCA diameters remained constant or increased slightly as transmural pressure was decreased. In contrast, both groups subjected to moderate fluid percussion TBI exhibited decreased MCA diameters as transmural pressure was decreased (Figure 9, TBI-5 and TBI-30). These results demonstrate that responses to changes in intravascular pressure are reduced in pressurized MCA segments. These studies suggest that, while responses to applied vasoconstrictor and vasodilator agents in ring segments from posterior and middle cerebral arteries are unaffected by TBI, MCA segments *ex vivo* behave more like intact cerebral arterioles *in vivo* (i.e., pial arterioles), perhaps because of the presence of an intact perivascular sensory nerve plexus.

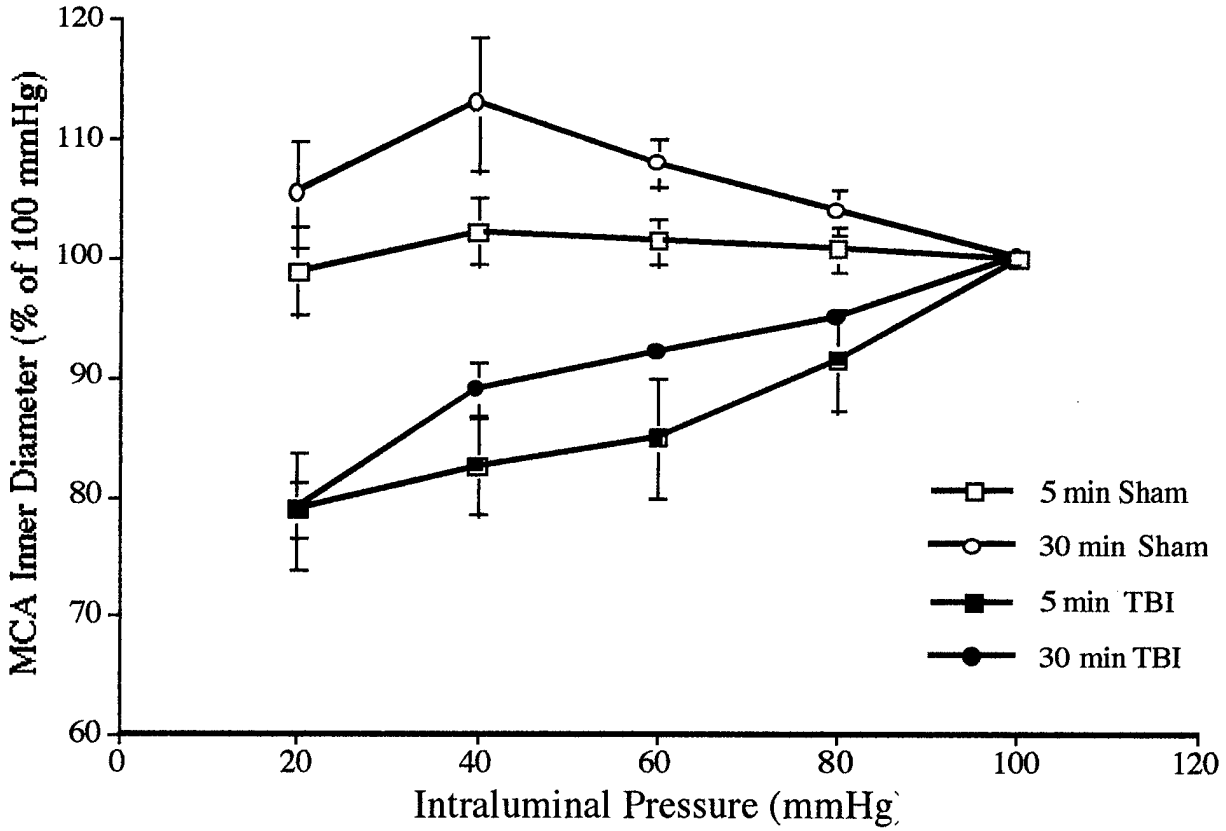


Figure 9. Diameter change in response to reductions in intravascular pressure in middle cerebral artery segments harvested 5 or 30 minutes after sham injury (sham-5; sham-30) or moderate TBI (TBI-5; TBI-30).

Specific Aim 2 *is to address the hypothesis that post-TBI cerebral hypoperfusion is caused by nitric oxide (NO)-dependent mechanisms. NO synthase (NOS) activity will be assessed post-TBI to determine its contribution to the vascular effects observed.*

The first set of experiments (SA 2.1 - CBF after fluid-percussion TBI in rats: treatment with L-arginine, D-arginine and superoxide dismutase [SOD]) demonstrates that L-arginine (but not the inactive stereoisomer, D-arginine) prevented and SOD reversed cerebral hypoperfusion after TBI. These studies, which are summarized below, have been published (8) (appendix).

The second set of experiments (SA 2.2 - Effects of NOS inhibition on CBF after TBI) describes experiments designed to test the hypothesis that, if TBI decreases CBF by reducing NO levels, then NOS inhibition after TBI should not produce further reductions in CBF.

The third set of experiments (SA 2.3 - Basal or stimulated NOS activity after TBI) tests the hypothesis that TBI reduces NO levels and CBF by decreasing NOS activity. Both basal (resting) NOS activity and NOS activity in response to stimulation were determined after TBI. These studies are described in detail in Alagarsamy, et al., (9) (appendix).

The fourth set of experiments (SA 2.4- Microdialysis measurement of cerebral nitric oxide levels) determines whether levels of NO_2/NO_3, stable metabolites of NO, could be measured in cerebral microdialysate from animals before and after treatment with L-nitro arginine methyl ester (L-NAME). If feasible, this method will be used to directly measure NO_2/NO_3 levels after TBI and L-arginine treatment.

The fifth set of studies (SA 2.5 - Myogenic responses after TBI: effects of treatment with L-arginine) address the hypothesis that TBI-induced reductions in NO might contribute to reduced vasodilatory responses to hypotension in isolated cerebral arteries after TBI and that the addition of L-arginine may restore these responses.

The sixth set of experiments (SA 2.6 - Effects of TBI on CGRP-containing perivascular nerve density) tests the hypothesis that a vasodilatory neurotransmitter other than NO might be involved in CBF reductions or in impaired cerebral vascular responses after TBI. Calcitonin gene-related peptide (CGRP), a vasoactive transmitter contained in nerves that surround the cerebral circulation, has been implicated in autoregulatory responses to hypotension (10). The density of CGRP-containing perivascular nerves was counted after moderate and severe TBI in order to determine whether TBI affected the number of these potentially regulatory nerve fibers.

The seventh set of experiments (SA 2.7 - Myogenic responses to hypotension: Possible role of anandamide and perivascular nerves) investigates another vasodilatory agent that might be important for the control of the cerebral vasculature normally as well as after TBI. These experiments demonstrate that the cannabinoid 1 receptors may be important in autoregulatory vasodilation and, therefore, may be damaged by TBI and/or subsequent hypotension. These studies are described in a manuscript which has been submitted to the *American Journal of Physiology* (manuscript in appendix).

<u>SA 2.1 - CBF after fluid-percussion TBI in rats: treatment with L-arginine and SOD.</u>

Rats anesthetized with isoflurane (1.5%) were prepared for moderate fluid percussion TBI and randomly assigned to receive a saline placebo (n=8) , L-arginine (100 mg/kg, i.v., 5 min post-TBI, n=8), SOD (24,000 U/kg, i.v. bolus before TBI + 1,600 U $\cdot$ kg^{-1} $\cdot$ min^{-1} for 10 min starting immediately after TBI, n=8), L-arginine + SOD (n=8) or sham-injury + L-arginine (n=8). CBF was measured by laser Doppler flowmetry (LDF) for 2 hours after TBI. In the rats treated with saline placebo, CBF decreased significantly after TBI (Figure 10), confirming previous microsphere (5,11) and LDF (12) reports of transient hypoperfusion that occurs after fluid-percussion TBI in rats. Rats treated with L-arginine without injury exhibited no change in CBF. The rats treated with L-arginine exhibited no CBF decreases, and the SOD-treated rats exhibited CBF decreases that resolved within 45 minutes of TBI (Figure 10) (13). A subsequent group of rats (n=5 per group) was prepared identically but received a saline placebo, L-arginine (100 mg/kg, i.v., 5 min post-TBI) or D-arginine (100 mg/kg, i.v., 5 min post-TBI). CBF decreased significantly in the groups treated with the placebo or D-arginine but did not change in the L-

arginine-treated group (Figure 11). These observations suggest that posttraumatic hypoperfusion is caused by NO destruction by O_2^-.

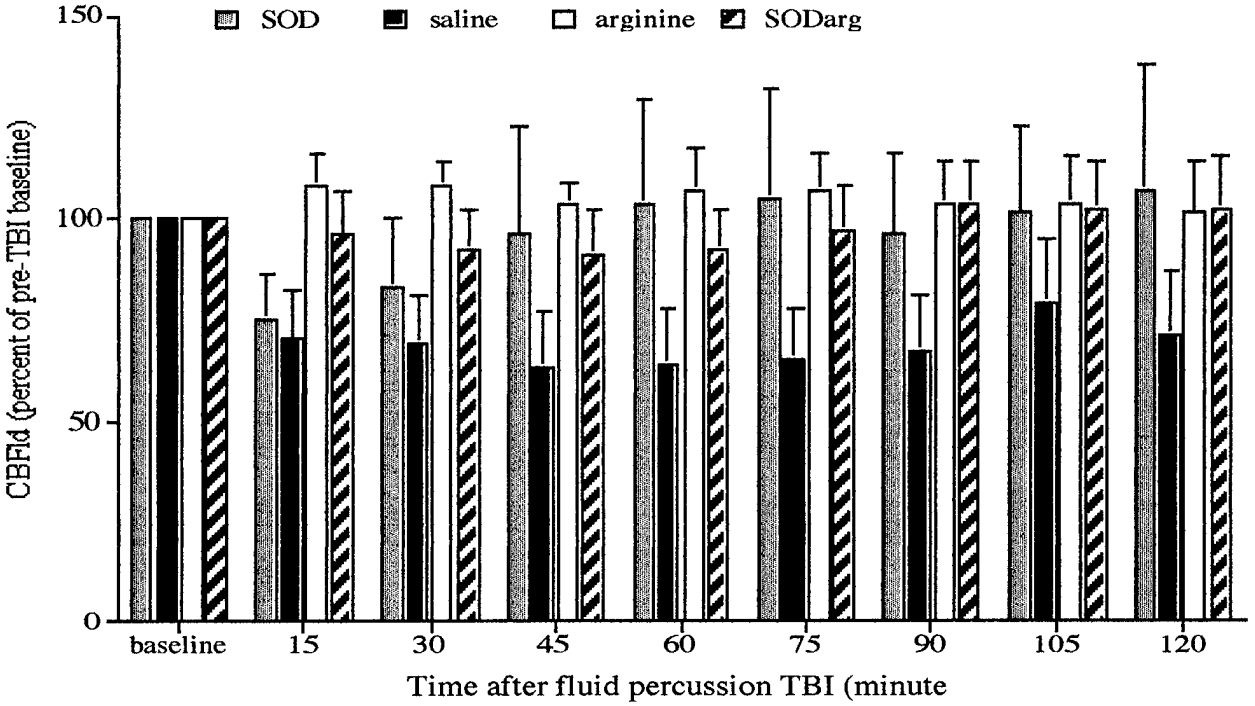


Figure 10. CBF_{LD} after moderate (2.2 atm) TBI in rats treated with SOD, saline placebo, L-arginine or SOD + L-arginine (SODarg).

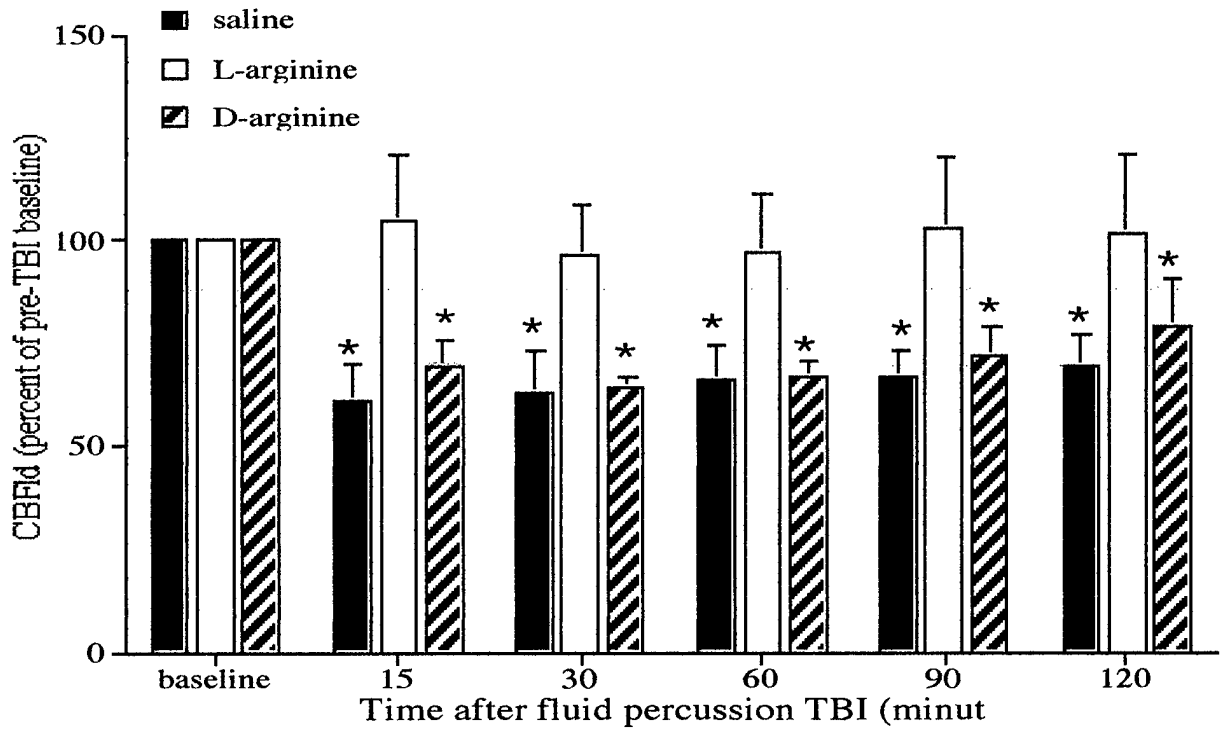


Figure 11. CBF_{LD} after moderate (2.2 atm) TBI in rats treated with L-arginine, saline placebo, or D-arginine.

SA 2.2 - Effects of NOS inhibition on CBF after TBI.

We reported that L-arginine, but not D-arginine, improves CBF after TBI in rats (8), suggesting that TBI reduces CBF by NO-related mechanisms. If TBI decreases CBF by reducing NO levels, then inhibition of NO synthesis should reduce CBF to levels similar to those observed after TBI. In addition, if TBI is inactivating NO, then NOS inhibition after TBI should not produce additional CBF decreases. To test these hypotheses, rats were treated with the NOS inhibitor L-NAME after TBI or sham-TBI. In a fourth group MAP was maintained constant during L-NAME infusion using an aortic reservoir. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch. Male Sprague-Dawley rats (400-450g) were anesthetized with 1.5-2% isoflurane in O_2:air (70:30), intubated, and ventilated before surgical preparation for midline fluid percussion TBI as described (14). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.8-mm hole was trephined into the skull over the sagittal suture and a modified Luer-Loc syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic and dental cement. In one group a polyethylene catheter (PE 205) was placed in the abdominal aorta and connected to a reservoir that could be raised or lowered to maintain arterial blood pressure, which was monitored via a polyethylene catheter in one femoral artery.

After surgery, isoflurane was lowered to 1.0-1.5%; the rats were connected to the trauma device, and subjected to moderate (2.2 atm.) TBI or sham-TBI. They were then randomly assigned to receive moderate TBI alone (TBI, n=5), sham-TBI followed 30 minutes later by a bolus of L-NAME (Sham+L-NAME, 30 mg/kg, i.v., n=5), moderate TBI followed 30 minutes later by a bolus of L-NAME (TBI+L-NAME, 30 mg/kg, i.v., n=5), or moderate TBI followed 30 minutes later by a bolus of L-NAME with controlled mean arterial blood pressure to prevent during L-NAME infusion (TBI+L-NAME+AB, 30 mg/kg, i.v., n=5). In all groups, arterial blood pressure and CBF were measured continuously for 120 minutes after L-NAME infusion. NOS inhibition and moderate TBI reduce CBF to similar degrees. After TBI, CBF returned towards baseline within 2 hours while CBF decreases due to NOS inhibition persisted for the 120-minute measuring period (Figure 12). The combination TBI and L-NAME reduced CBF more than either treatment alone. While the observation that L-NAME and TBI reduce CBF to similar levels suggest that both reduce NO to a similar degree, the observation that NOS inhibition further reduces CBF suggests that some NO-mediated vasodilation remains after TBI. Further studies with more complete NOS inhibition, preferably with specific NOS inhibitors, are required to more accurately address the role of NO in posttraumatic hypoperfusion.

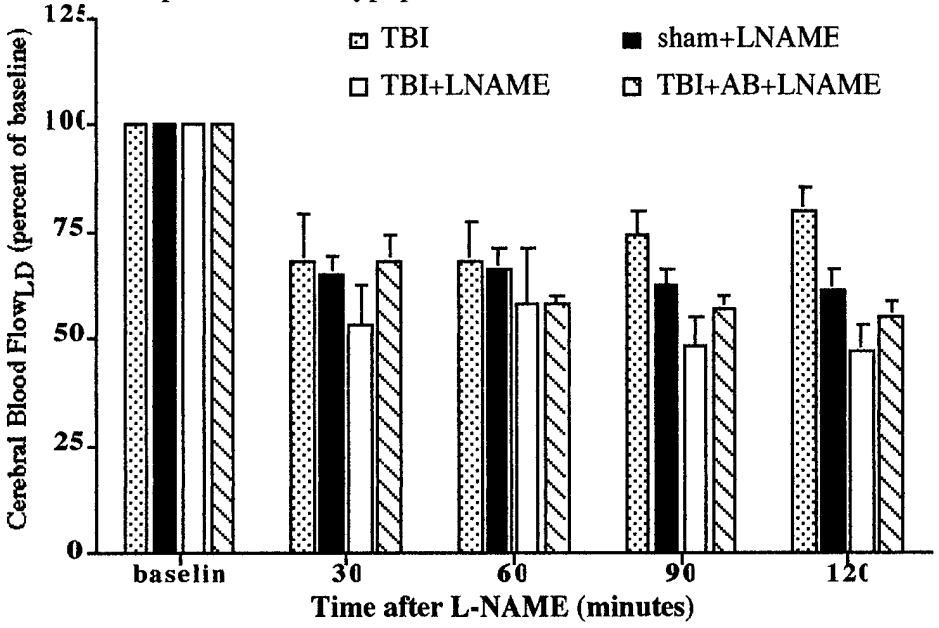


Figure 12. CBF LD after moderate (2.2 atm) TBI in rats treated with a saline placebo (TBI), sham-TBI + L-NAME (Sham+L-NAME), TBI + L-NAME (TBI+L-NAME), TBI followed 30 minutes + L-NAME with controlled arterial blood pressure (TBI+L-NAME+AB).

SA 2.3 - NOS activity after TBI.

To determine whether TBI would alter total NOS activity, assays of the conversion of arginine to assays of citrulline were made in crude enzyme preparations as described by Bredt and Snyder (15). Briefly, rats were decapitated and their brains were removed homogenized, the homogenate was centrifuged for 5 minutes at 3000 x g, and the supernatant was spun at 20,000 x g for 15 minutes. The supernatant from the second spin was passed over an ion exchange column to remove endogenous arginine. Activity of the NOS was monitored by measuring the levels of [3H]citrulline. Measurable NOS activity exists in preliminary experiments without the addition of exogenous tetrahydrobiopterin; thus this cofactor was not routinely added. These data are presented as amount of [3H]citrulline divided by the amount of [3H]arginine + [3H]citrulline ¥ 100/mg of protein.

There were no significant differences in NOS activity among control (n=10, unoperated), sham (n=5, prepared for TBI but not injured) or injured [n=5, moderate (2.2 atm) fluid-percussion TBI] rats at either low (0.45 μM) or high (1.50 μM) added L-arginine levels (Figure 13).

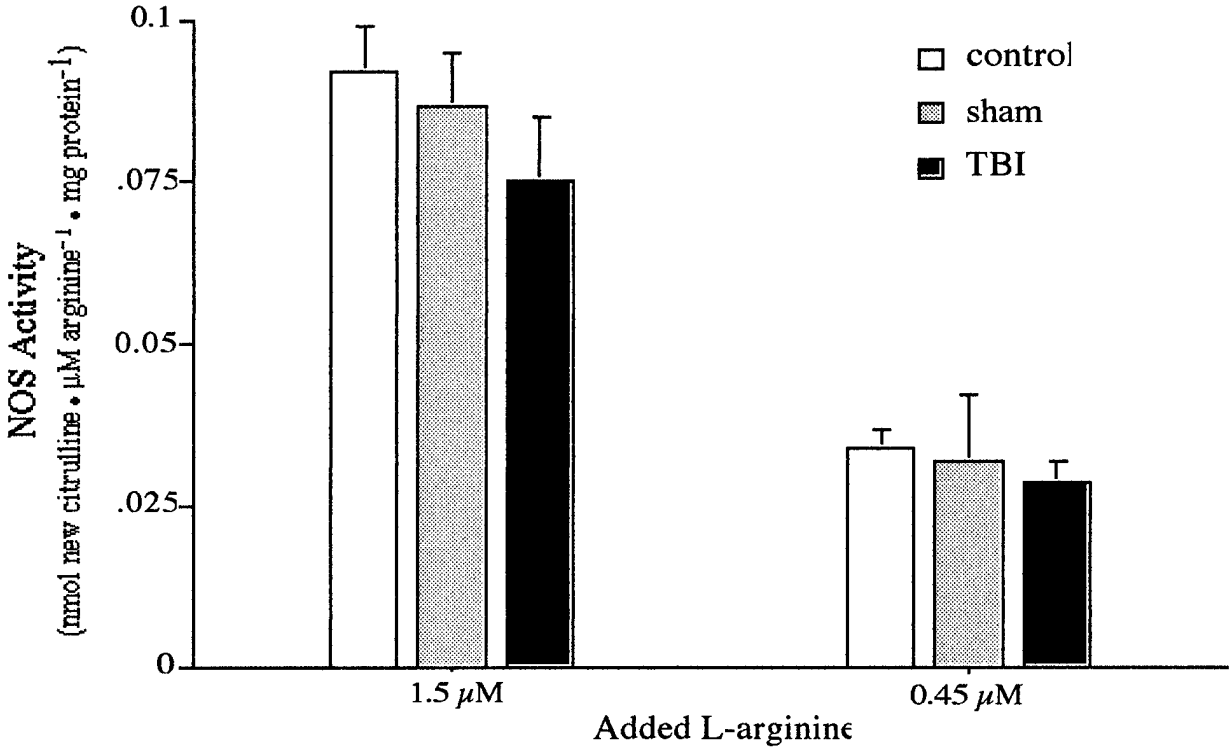


Figure 13. Total baseline soluble nitric oxide synthase (NOS) activity determined from crude enzyme preparations from control (unoperated, n=10) rats and rats subjected to sham injury (n=5) or moderate (n=5) fluid percussion TBI.

In order to determine whether stimulated NOS activity was affected by TBI, NOS activity was measured in minced tissue preparations using our modification (16) of previously described methods (15). After moderate TBI, rats were decapitated and the frontal cortices were dissected, cross-chopped and minced into slices (450 x 450 μm) . The conversion of L-[3H]-arginine to L-[3H]-citrulline in the cortical slices was used as an index of NOS activity. The methods of Bredt and Snyder (15) were modified to the extent that we used a 5-minute stimulation period. NOS activity in response to stimulation with N-methyl-D-aspartate NMDA (μM), a calcium ionophore (inomycin, 10 μM), or K+ (50 mM) was determined in injured (2.2 atm TBI) or sham-injured rats.

There was no difference in NOS activity in response to stimulation with NMDA (300 μM), K+ (50 mM) or ION (inomycin,10 μM) in control rats (n=9, no surgery) or in rats after moderate TBI (n=5, 2.2 atm) or sham-injury (n=5, preparation for TBI but no injury)(Figure 14).

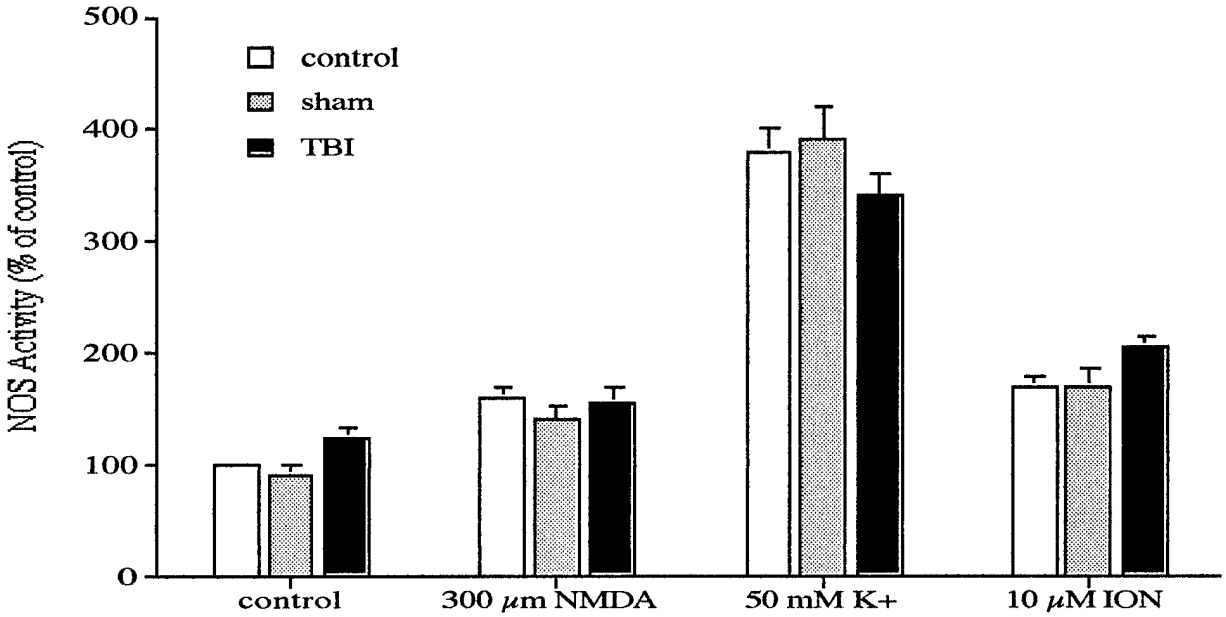


Figure 14. Stimulated cell-dependent NOS activity in mince preparations from control (unoperated, n=9) rats and rats subjected to sham injury (n=5) or moderate fluid percussion TBI (n=5) and then exposed to N-methyl-D-aspartate (NMDA), potassium chloride (K+), or ionomycin (ION).

SA 2.4- Microdialysis measurement of cerebral nitric oxide levels

In order to determine whether the NOS inhibitor L-NAME reduces cerebral NO levels, cerebral microdialysis was used to harvest samples that were analyzed for NO_2/NO_3, stable metabolites of NO, using an Antek 7020 NO Detector.

Rats (n=4) were anesthetized, intubated, and mechanically ventilated and maintained on 1.5-2.0% isoflurane. Cannulae were placed in femoral arteries and veins as described above. The rats were then placed in a stereotaxic head holder and a midline scalp incision was made. A craniotomy was made to the right of the sagittal suture between the lambda and bregma and the dura was nicked with a 25 gauge needle. The microdialysis probe was prepared according to the manufacturers instructions, and recovery rate was determined using a 5% dextrose solution at 37°C. The probe was then positioned 3.6 mm posterior and 1.5 lateral to the intersection of th bregma and the sagittal suture and inserted to a depth of 4 mm. The perfusion rate was set at 2μL/min, and the probe was allowed to equilibrate for 30 minutes. Samples were then collected every 20 minutes. Temporalis temperature, arterial blood pressure and blood gases and pH were maintained within normal limits during the sampling period. After 60 minutes of sampling, L-NAME (100 mg/kg, i.v.) was administered and samples were collected for an additional 60 minutes. Samples were collected and analyzed on the same day using an Antek 7020 NO Detector with an Antek Model 745 NO_2/NO_3 reduction assembly and a Model 742 Data Handling Software package.

Baseline NO_2/NO_3 levels were stable for the 60-minute pre-L-NAME sampling period (Figure 15). L-NAME administration resulted in a significant increase in mean arterial blood pressure. NO_2/NO_3 levels decreased approximately 50% after L-NAME administration. These studies indicate that microdialysis yields stable cerebral levels of the NO metabolites that decrease with NOS inhibition. This method will be used to monitor changes in NO levels after TBI with and without treatment with L-arginine to determine whether the effects of L-arginine on CBF after TBI are mediated by changes in NO.

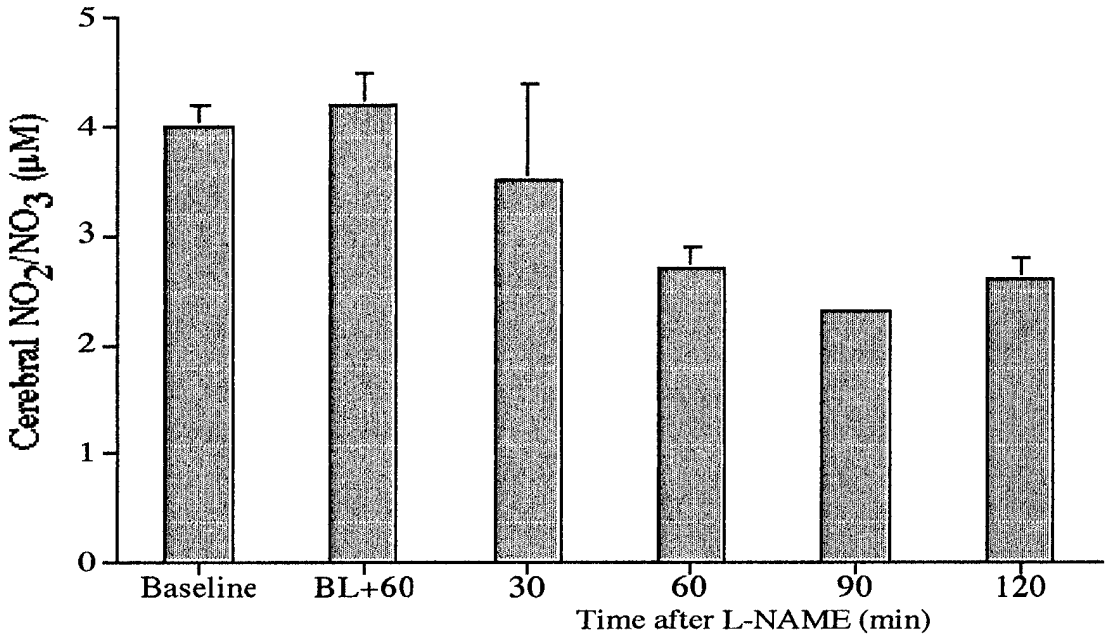


Figure 15 - NO_2/NO_3 levels in the rat cerebral cortex measured using microdialysis.

SA 2.5 - Myogenic responses after TBI: effects of treatment with L-arginine

We have reported that L-arginine (100mg/kg) completely prevents posttraumatic hypoperfusion in rats (8). In addition, we have reported that TBI reduces myogenic responses in isolated, pressurized MCA segments in rats harvested 5 or 30 minutes after moderate, central fluid percussion TBI (3). In order to determine whether L-arginine would improve myogenic responses, male Sprague-Dawley rats weighing 350-400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O_2:room air (70:30) using a volume ventilator. Polyethylene cannulae were placed in a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer and maintained using a thermostatically controlled water blanket. Rats were prepared for midline fluid-percussion TBI as previously described (14). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between the lambda and bregma and a modified LuerLok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was lowered to 1.5%; the rats were connected to the trauma device and randomly assigned to one of the groups listed below.

After TBI or sham injury, anesthetized rats were decapitated, their brains were removed, and their MCAs were harvested. Cerebral arteries were mounted in an arteriograph as described (17). Briefly, a section (2 mm) of the artery was mounted in the arteriograph by inserting micropipettes into the lumen at either end and securing the vessel with nylon suture (10 0). The mounted arterial segments were bathed in physiological salt solution (PSS) of the following composition: mM : NaCl, 130; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.17; glucose, 5; $CaCl_2$, 1.50, $NaHCO_3$, 15. When gassed with a mixture of 95% air and 5% CO_2, this solution has a pH of 7.4. After mounting, the PSS was warmed from room temperature to 37°C, and the arterial segments were allowed to equilibrate for 60 minutes with transmural pressure set at 50 mm Hg by raising reservoir bottles connected to the micropipettes. A pressure transducer between the micropipettes and the reservoir bottles was used to monitor transmural pressure across the arterial segment. The vessels were magnified with an inverted microscope equipped with a video camera and a monitor. Arterial diameter was measured using a video scaler calibrated with an optical micrometer. Before

testing for myogenic responses to changes in transmural pressure, contractility and endothelial function were tested using 30 mM K^+ or ACh (10^{-5} M), respectively. Myogenic responses were tested by increasing or decreasing luminal pressure in 20 mm Hg increments with a 5-minute equilibration period at each pressure level before diameter measurements were made. After the initial myogenic responses were evaluated, the PSS was replaced with calcium-free PSS and myogenic responses were assessed again.

Rats were assigned to one of the following three groups (n=5 rats per group):
Sham-TBI - MCAs were harvested 5 minutes after sham injury.
TBI - MCAs were harvested 5 minutes after moderate, central fluid percussion TBI.
TBI-arg - Rats were treated with L-arginine (100 mg/kg) before TBI and then MCAs were harvested 5 minutes after TBI.

Sham-injured rats exhibited normal vasodilatory responses to reductions in transmural pressure (Figure 16). After TBI, MCA diameters decreased progressively with each reduction in transmural pressure. Rats treated with L-arginine showed less reduction in MCA inner diameters but the responses in arteries from the treated rats were not significantly different than responses in arteries harvested from untreated rats. These studies, indicating that L-arginine does not preserve myogenic responses after TBI, are consistent with previous reports indicating that CBF autoregulation is not affected by NOS inhibitors in rats (18).

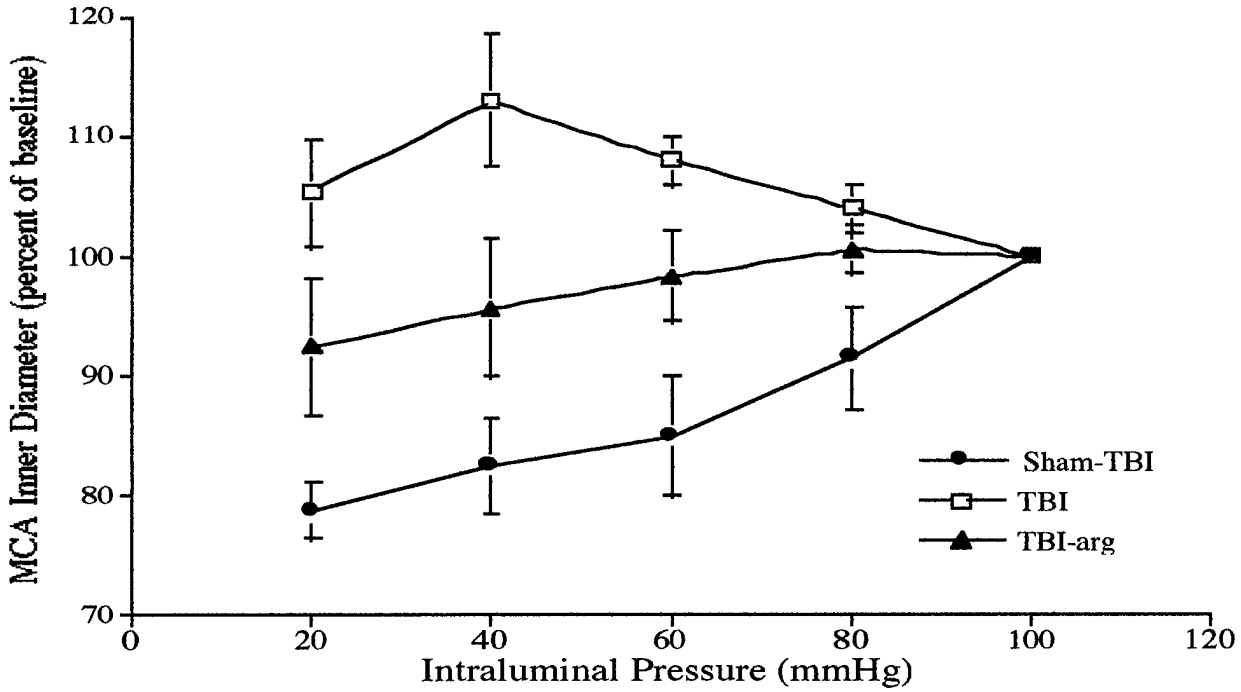


Figure 16 - Inner diameters in MCAs harvested from rats after sham-injury (Sham) or moderate TBI (TBI) or TBI and treatment with L-arginine (100mg/kg).

SA 2.6 - Effects of TBI on CGRP-containing perivascular nerve density

Inhibition of CGRP receptors has been reported to reduce autoregulatory responses to hypotension in rodents. We tested the hypothesis that TBI impairs cerebral autoregulation by reducing CGRP-containing perivascular nerve density. In order to test this hypothesis, we determined whether TBI reduced the number of CGRP-containing perivascular nerve fibers, which were measured using immunohistochemical staining of CGRP and stereologic counting.

Using a protocol approved by the IACUC of The University of Texas Medical Branch, male Sprague-Dawley rats (450-550 g) were anesthetized, intubated, ventilated on 1.5-2.0% isoflurane in air:O2 (70:30), and prepared for midline fluid percussion TBI as previously described (8). Cannulae were placed in a femoral artery and vein for monitoring of arterial blood pressure

and drug infusion, respectively. Isoflurane was then lowered to 1.5% and rats were randomly chosen to receive a sham TBI or moderate (2.0 atm) or severe (3.0 atm.) TBI. Following the TBI, isoflurane was increased to 2.0% and the rats were perfused transcardially with normal saline in order to remove the blood. The cerebral arterial circle of Willis and its large branches were removed and stretched flat on gelatin-treated microscope slides and fixed for 10 minutes in ice cold methanol. After rinsing in Tris-buffered saline, intrinsic peroxidase activity was blocked using a commercial peroxidase blocker (DAKO, Inc.) and non-specific protein binding was blocked by incubation with a serum-free blocker (DAKO, Inc.). The slides were then incubated at 4°C overnight with polyclonal anti-CGRP (1:500) and then with the biotin-conjugated secondary antibody. The antigen-antibody complex was visualized using commercially available stains, dehydrated, and permanently mounted. Nerve density was determined using stereologic methods (19). Briefly, analysis of all the CGRP-containing nerves in each section was made using photomicrographs of the vessel segment taken at different planes of focus. Intersections of nerves with a plastic grid were then counted by a blinded observer and the density as length/area was calculated at $\pi N/2L$, where N is the number of intersections and L is the total grid length.

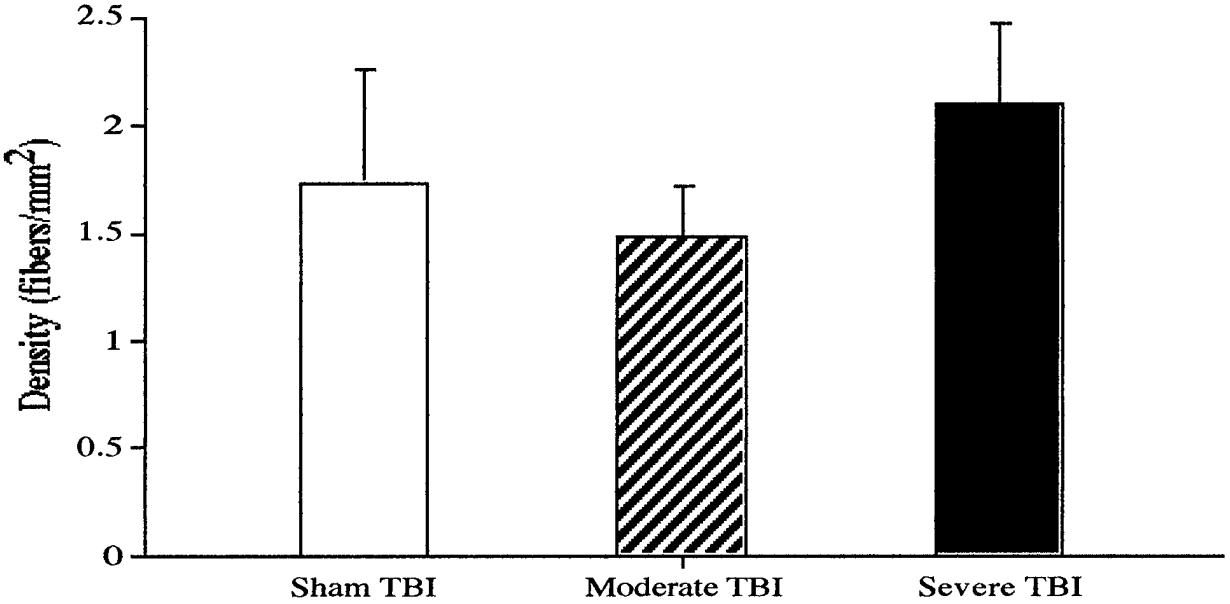


Figure 17 - Density (fibers/mm^2) of CGRP immunoreactive perivascular nerve fibers harvested from rats after sham-TBI (Sham) or moderate (Mod. TBI) or severe (Sev. TBI) TBI. Values are mean ± SEM.

There was no significant difference in CGRP-containing perivascular nerve density in rats after low or high levels of central fluid percussion injury (Figure 17). These studies suggest that CGRP-containing perivascular nerves are not affected by TBI and, therefore, are probably not involved in TBI-induced impairment of autoregulation However, it is possible that TBI affects other vasodilatory agents that may be contained in perivascular nerves.

SA 2.7 - Myogenic responses to hypotension: Possible role of anandamide and perivascular nerves.

In addition to these known perivascular neurotransmitters, other agents have been reported to be cerebral vasodilators. One such agent is anandamide, an endogenous ligand of the CB1 receptor. Anandamide is a potent cerebral vasodilator (20), and we have exciting new evidence that inhibition of the anandamide receptor reduces myogenic responses in isolated pressurized cerebral arteries. Rats were anesthetized with isoflurane and decapitated, and their MCAs were harvested, mounted in the arteriograph, and allowed to equilibrate as described above. Changes in MCA diameters were measured as intraluminal pressure was reduced progressively from 100 to 20 mm Hg in 20 mm Hg increments. In the first group of rats (n=5) myogenic responses were tested

twice with approximately 30 minutes in between tests. In this time control group, myogenic responses were similar between the first and second test (data not shown). A second group of rats (n=12) was treated identically except that the vessels were filled with SR141716A, a specific antagonist of the CB1 receptor (3μM, n=6), or with the ethanol vehicle (n=6). In this group, the ethanol vehicle had no effect on myogenic responses (data not shown) but myogenic responses were reduced significantly compared to the pre-treatment responses in the SR141716A-treated arteries (Figure 18).

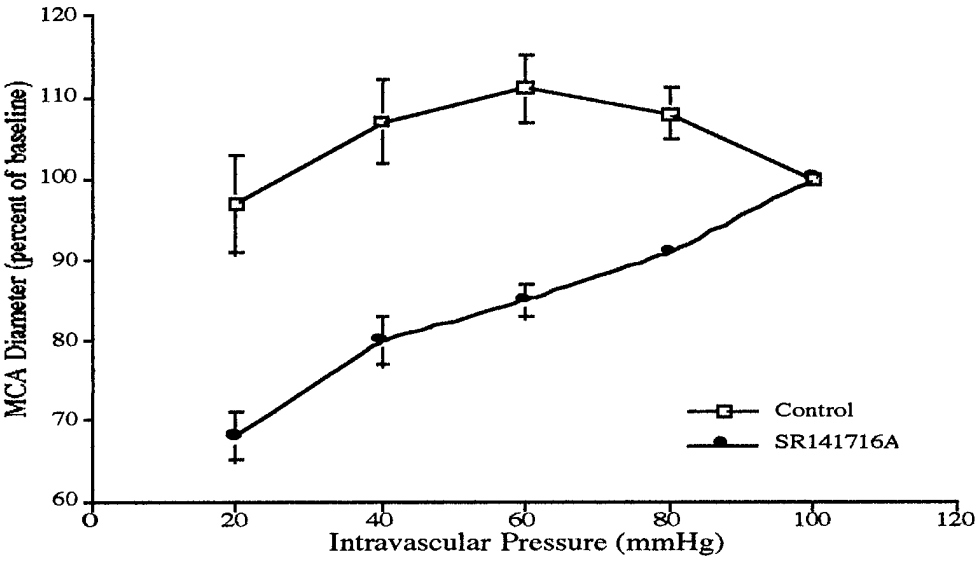


Figure 18 - Inner diameters in MCA's before (Control) and after (SR141716A) treatment with SR141716A (3μM).

A fourth group of rats was prepared identically to the other three groups but were treated with extraluminal application of phenol (0.5%) for 10 minutes in between the two evaluations of myogenic responses. Phenol treatment did not affect vasoconstrictor responses to serotonin but significantly reduced myogenic responses to progressive reductions in intraluminal pressure (Figure 19). Phenol, when applied at the concentrations and times used in this study, destroys adventitial perivascular nerve fibers without affecting the underlying vascular smooth muscle or endothelium.

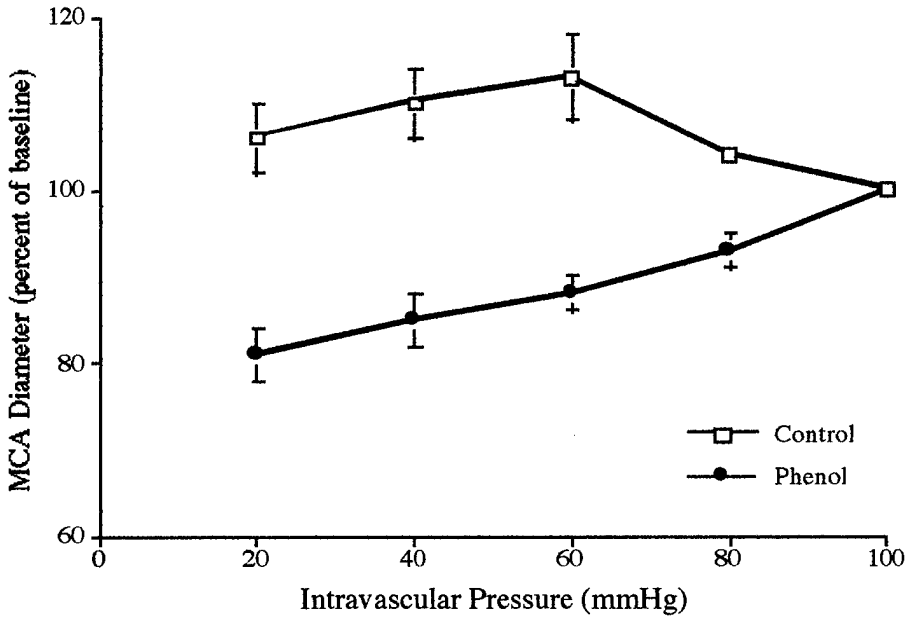


Figure 19 - Inner diameters in MCAs before (Control) and after (Phenol) extraluminal treatment with phenol (0.5% for 10 min).

These studies suggest that perivascular nerve fibers may be involved in vasodilatory responses to hypotension, perhaps by the action of an endogenous cannabinoid ligand such as anandamide.

Specific Aim 3 *is to address the hypothesis that increased production of $\cdot O_2^-$ during TBI and subsequent hypotension/resuscitation is responsible for the impaired cerebrovascular reactivity.*

The first set of experiments under Specific Aim 3 (3.1 - Superoxide radical production and CBF after TBI) tests the hypothesis that treatment of rats with a free radical scavenger after TBI would reduce $\cdot O_2^-$ levels and improve CBF. The results of these experiments, which have been published (21) (appendix), are summarized below.

The second set of studies (SA 3.2 - CBF responses to controlled hemorrhagic hypotension (autoregulation) after TBI: effects of SOD treatment) examine the hypothesis that treatment with SOD would improve cerebral vasodilatory responses to hypotension (i.e., autoregulation) after TBI.

Hypothermia is known to be protective in TBI and cerebral ischemia, but the mechanisms are not well understood. Hypothermia reduces damage due to oxygen free radicals in a variety of situations (22,23), and oxygen radical scavengers restore cerebral vascular reactivity after TBI (24). Therefore, in this third set of experiments (SA 3.3 - Effects of hypothermia on CBF after TBI) we hypothesize that hypothermia might improve autoregulation after TBI. Evidence that hypothermia, which reduces $\cdot O_2^-$ production and oxidative damage, preserved autoregulation would be consistent with the hypothesis that $\cdot O_2^-$ production contributes to the pathophysiology of TBI.

The fourth set of investigations (SA 3.4 - Nitrotyrosine immunoreactivity after TBI and hemorrhagic hypotension) explores the hypothesis that part of the damage caused by $\cdot O_2^-$ after TBI is actually due to $\cdot O_2^-$ reacting with NO to form the powerful oxidant, peroxynitrite ($ONOO^-$). To determine whether $ONOO^-$ is formed after TBI and hypotension, cerebral arteries were examined for nitrotyrosine immunoreactivity after TBI, hemorrhagic hypotension and resuscitation. Nitrotyrosine is formed when peroxynitrite nitrosylates tyrosine residues in proteins (25,26).

The fifth set of investigations (SA 3.5 - Effects of peroxynitrite on myogenic responses in isolated, pressurized middle cerebral arteries) addresses the hypothesis that the $ONOO^-$ produced by $\cdot O_2^-$ and NO after TBI contributes to reduced cerebral vasodilatory responses to hypotension. These studies are described in detail in a manuscript which has been submitted to *Stroke* (27) (manuscript in appendix).

The sixth group of experiments (SA 3.6 - Effects of $ONOO^-$ or TBI on cerebral vasodilatory responses *in vitro*) further explores the hypothesis that $ONOO^-$ or TBI reduce cerebral vasodilatory responses to CGRP or to chromakalim, an activator of K^+ channels in vascular smooth muscle. CGRP has been implicated in autoregulatory responses to hypotension (10) and K^+ channels appear to contribute to cerebral vasodilatory responses to NO and hypoxia (28).

3.1 - Superoxide radical production and CBF after TBI

CBF and $\cdot O_2^-$ production were measured in rats treated with an experimental free radical scavenger and inhibitor of lipid peroxidation (U-74389G, 16-desmethyl-tirilizad, Upjohn/Pharmacia) in order to determine whether changes in $\cdot O_2^-$ and CBF were related. Using a cytochrome C-coated platinum electrode, we measured $\cdot O_2^-$ production in isoflurane anesthetized rats after moderate TBI (29). Superoxide levels increased after TBI and local and systemic injection of SOD transiently reduced $\cdot O_2^-$ level. In a second series of rats, CBF and $\cdot O_2^-$ levels were measured simultaneously. Cytochrome C electrodes were constructed of platinized carbon electrode (PACE) material and calibrated for sensitivity to $\cdot O_2^-$ using the xanthine-xanthine oxidase system. Rats (n=12) were anesthetized with isoflurane, intubated, mechanically ventilated with 1.5-2.0% isoflurane, and prepared for moderate fluid-percussion TBI (2.2 atm); recordings of $\cdot O_2^-$ production were made using an electrode positioned 2 mm lateral to the midline and anterior to bregma using a DCV-5 potentiostat (E'-Chem, BAS). A counter-electrode was positioned

contralaterally, and the reference electrode was attached to the skin. Recordings were made with an applied voltage of 0.05mV relative to a saturated mercury-calamine electrode. Relative CBF was measured using LDF probe placed over the thinned calvaria contralateral to the injury site. After surgical preparation, isoflurane was lowered to 1.5% and CBF was monitored continuously and expressed as mean ± SEM percent of preinjury perfusion. Rats were treated with either U-74389G (3mg/kg, n=6) or a carrier (20 mM sodium citrate, 77 mM NaCl, n=6) and subjected to moderate fluid percussion TBI. An additional group of rats (n=3) was prepared identically but the PACE electrode was coated with bovine serum albumin instead of cytochrome C. CBF and O_2^- production were measured for 90 minutes after TBI.

After TBI in both the U-74389G and carrier-treated groups, CBF decreased and remained below baseline for the 90-minute measurement period (Figure 20). In the carrier-treated group, $\cdot O_2^-$ levels rose to a peak at about 30 minutes after TBI (Figure 20, open circles) and then remained elevated for the 90-minute measurement period. $\cdot O_2^-$ rose in the U-74389G-treated group after TBI, but to significantly lower levels and then returned to baseline levels within 80 minutes of TBI (Figure 21, closed circles). There was no change in signal after TBI in the group with the electrode that was not coated with cytochrome C (Figure 21, closed triangles). Interestingly, there was a disassociation between $\cdot O_2^-$ levels, which decreased in the U-74389 group, and CBF, which was not affected by treatment. These observations suggest that either increases in $\cdot O_2^-$ do not contribute to posttraumatic hypoperfusion or that the increases in $\cdot O_2^-$ that occurred after TBI in the U-74389G-treated rats were sufficient to inactivate NO and produce cerebral hypoperfusion.

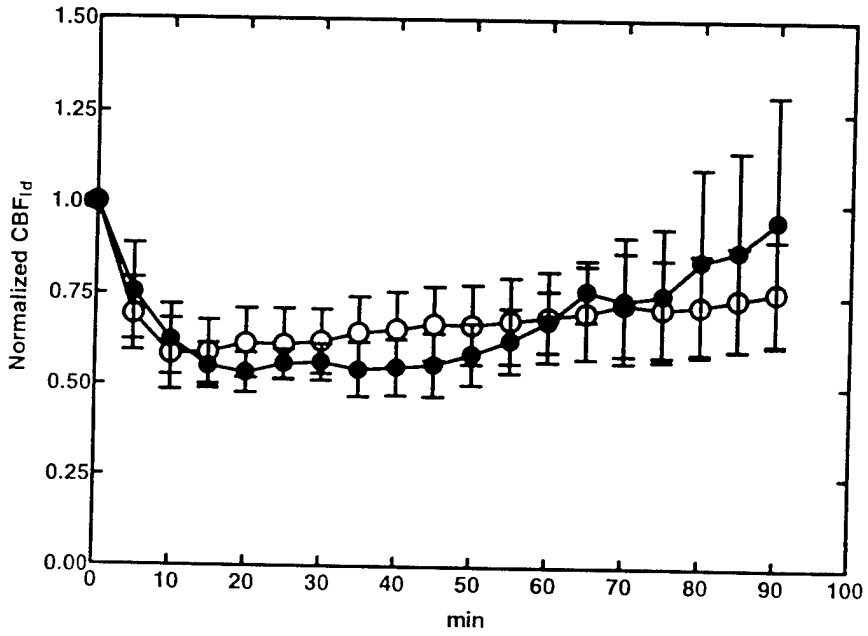


Figure 20. CBF_{LD} measurements after moderate (2.2 atm) TBI in rats treated with a carrier (closed circles, n=6) or with U-74398G (open circles, n=6). CBF_{LD} measurements are percent of pre-TBI baseline.

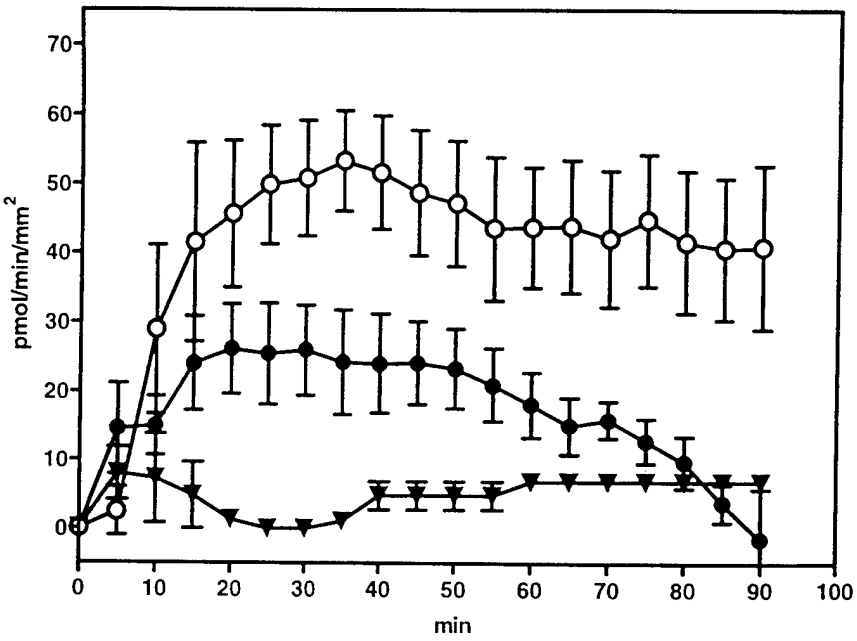


Figure 21. $\cdot O_2^-$ measurements after moderate (2.2 atm) TBI in rats treated with a carrier (closed circles, n=6) or with U-74398G (open circles, n=6). An additional group of rats (n=3) was prepared identically, but the PACE electrode was coated with bovine serum albumin instead of cytochrome C (closed triangles).

SA 3.2 - CBF responses to controlled hemorrhagic hypotension (autoregulation) after TBI: effects of SOD treatment

Using LDF in isoflurane-anesthetized rats, CBF was recorded during periods of hemorrhagic hypotension before and after TBI. Hypotension was produced by bleeding into a reservoir connected to the abdominal aorta via a polyethylene cannula placed distal to the renal arteries. Raising or lowering the reservoir produced steady changes in MAP in 10 mm Hg increments. An autoregulatory challenge was performed before injury and then at 30-minute intervals for 120 minutes after TBI. In untreated rats (n=4), autoregulation was impaired after TBI, as demonstrated by greater decreases in CBF during hypotension after TBI than before TBI. Impaired autoregulation persisted for 120 minutes after TBI. In rats treated with SOD [24,000 U/kg, i.v. bolus before TBI + 1,600 U · kg^{-1} · min^{-1} for 10 min starting immediately after TBI (n=5)], CBF during hypotension was improved at 30 minutes post-TBI (Figure 22) and was restored to pre-TBI levels at 90 minutes post-TBI (Figure 23).

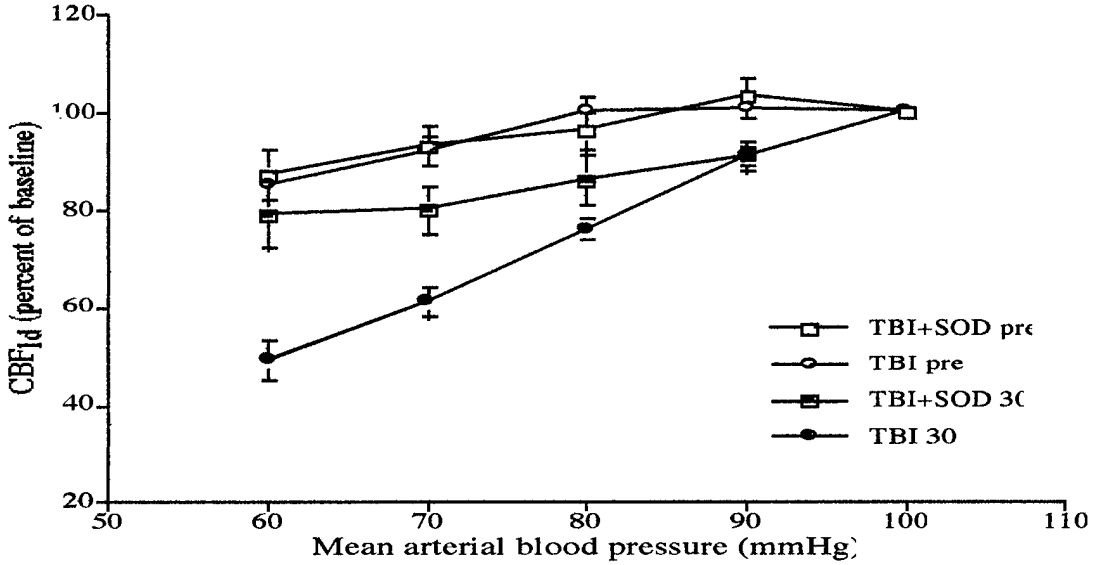


Figure 22. CBF_{LD} during hemorrhagic hypotension 30 minutes after moderate TBI (2.2 atm) in rats untreated (n=4) or treated with SOD (n=5).

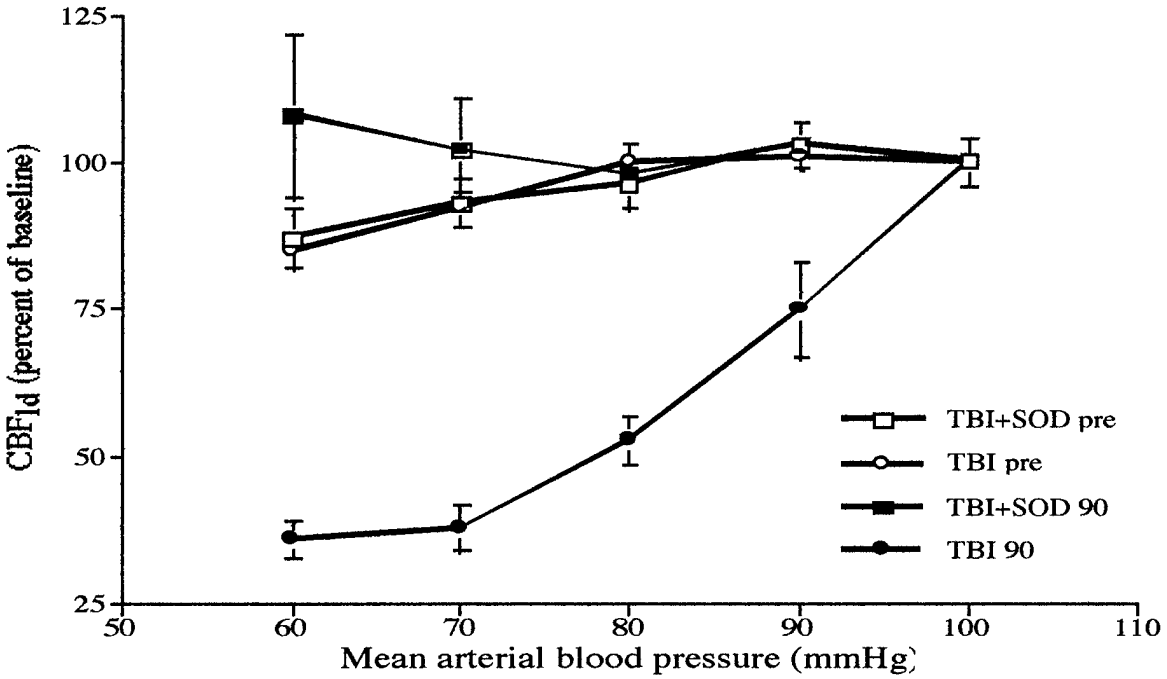


Figure 23. CBF_{LD} during hemorrhagic hypotension 90 minutes after moderate TBI (2.2 atm) in rats untreated (n=4) or treated with SOD (n=5).

SA 3.3 - Effects of hypothermia on CBF after TBI

Mild hypotension worsens outcome after TBI in humans (30), perhaps because of TBI-induced impaired cerebral vasodilation (31). Hypothermia improves outcome after TBI in animals (32) and humans (33), but the mechanism remains unclear. This study evaluated the effects of hypothermia on CBF pressure autoregulation after FPI in rats.

In an approved protocol, 31 Sprague-Dawley rats were anesthetized with isoflurane, intubated, mechanically ventilated with 1.5-2% isoflurane in 2:1 air:oxygen, and prepared for right lateral fluid percussion TBI and cerebral LDF. A 4.8-mm trephine craniotomy was created midway between the sagittal suture and the insertion of the temporalis muscle and midway between the lambda and bregma followed by insertion of machined TBI adapter. The skull was thinned with an air-cooled dental drill over the left parietal lobe midway between the sagittal suture and the insertion of the temporalis muscle and midway between the lambda and bregma for placement of the LDF probe. The animal was turned supine, and, via a mid-line abdominal incision, the aorta was exposed at the level of the iliac bifurcation, cannulated with PE210 tubing, heparinized with 50 units heparin, and connected via silastic tubing ,to a large volume variable height reservoir. The reservoir was primed with 20 ml of donor rat whole blood (0.5cc CPD solution and 12.5 units heparin for each 10cc of whole blood) and a timer was begun. The animal was placed prone in a stereotaxic head holder, the FPI device attached via a 60-cm length of non-compliant tubing, the LDF probe was placed, and the bilateral temporalis muscle temperature probes were attached. Temperature was maintained at 37 ± 0.5° C using heating lamps, heating blanket, fans, and iced isopropyl alcohol as needed. Isoflurane was decreased to 1%, and baseline arterial blood gas and hematocrit were obtained. Mechanical ventilation was adjusted to maintain a $PaCO_2$ of 33-38 mm Hg without correction for temperature (alpha-stat management) throughout the study. CBF autoregulation was obtained by measuring changes in LDF at various arterial blood pressures. The systemic arterial blood pressure was measured at the junction of the aorta and the silastic tubing, while the blood pressure was manipulated by raising and lowering the reservoir height with respect to the animal. LDF was measured at a MAP of 100 mm Hg (used as the baseline blood pressure and CBF) followed by randomly assigned changes in blood pressure to either 80, 60, or 40 mm

Hg. After stabilization of the blood pressure and LDF signal at each blood pressure (120 to 240 seconds) MAP and CBF were recorded and the blood pressure was returned to 100 mm Hg for a 2-3 minute restabilization period. The process was repeated until all pressure perturbations were completed. After baseline autoregulation testing at 37° C, the animals were randomized to one of 5 groups:

37° C without FPI (Control 37)
37° C with FPI of 1.8 atm (FPI 37)
32° C without FPI (Control 32)
32° C with FPI 1.8 atm (Pre-FPI 32)
37° C with FPI 1.8 atm and immediate cooling to 32° C (Post-FPI 32).

After collecting arterial blood gases and hematocrits, the correct temperature was established and the appropriate FPI delivered. Cerebral autoregulation measurement was repeated at 30 and 60 minutes after FPI. At the completion of the study, the animals were killed with saturated KCl. Individual animal exclusion criteria included: study time greater than 120 minutes from aortic occlusion to study completion, hematocrit less than 34, pH of < 7.3 with $PaCO_2$ in the target range, PaO_2 < 100 mm Hg, or death of the animal during the preparation.

There were no significant differences among groups with respect to animal weight, $PaCO_2$, pH, PaO_2, or hematocrit. At baseline, no significant differences in CBF were observed among groups at any level of MAP (Figure 24). CBF changes occurred in the TBI and TBI + hypothermia groups at 30 and 60 minutes (Figures 25 & 26). There were no significant differences in CBF changes between the normothermic TBI (FPI 37) and the TBI + hypothermia (Pre-FPI 32 and Post-FPI 32) groups. These studies indicate that hypothermia initiated prior to or following TBI does not lead to improvements in CBF when compared to normothermic TBI. Therefore, the protective mechanisms of hypothermia are most likely related to mechanisms other than the restoration of CBF after TBI.

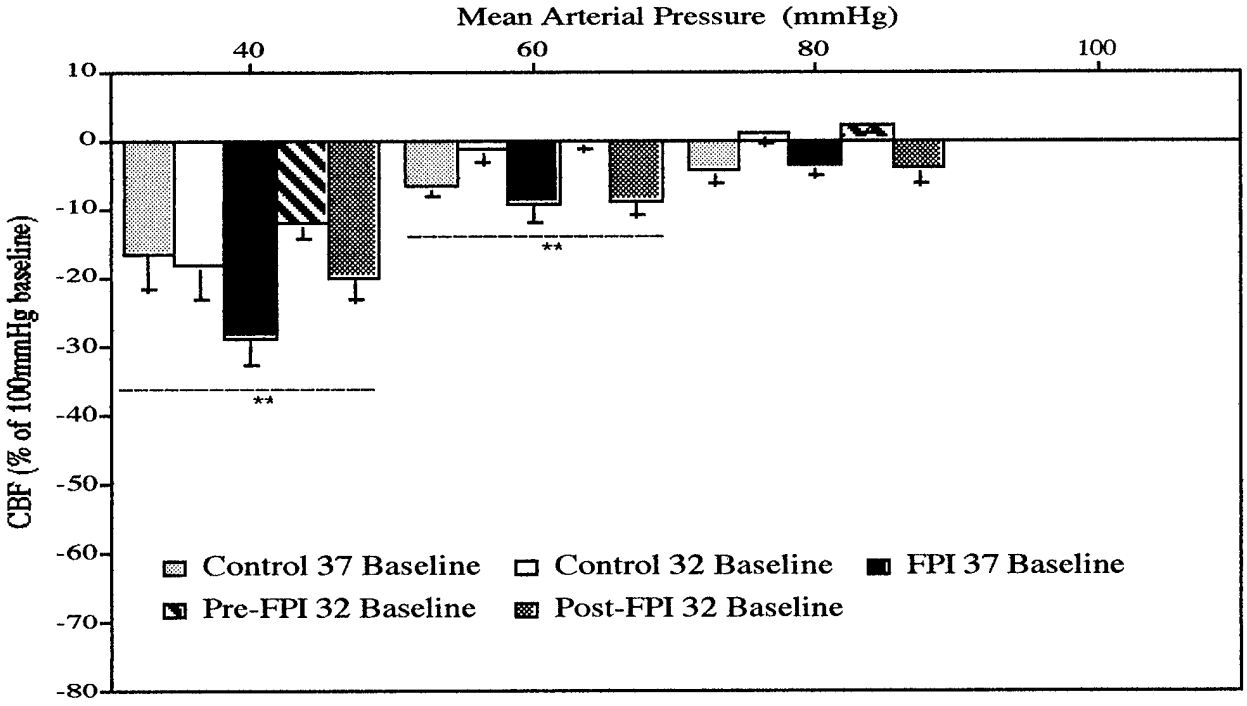


Figure 24 - Comparison of percent changes in CBF (compared to 100 mm Hg baseline) across groups prior to TBI. ** = $p < 0.05$ compared to baseline CBF.

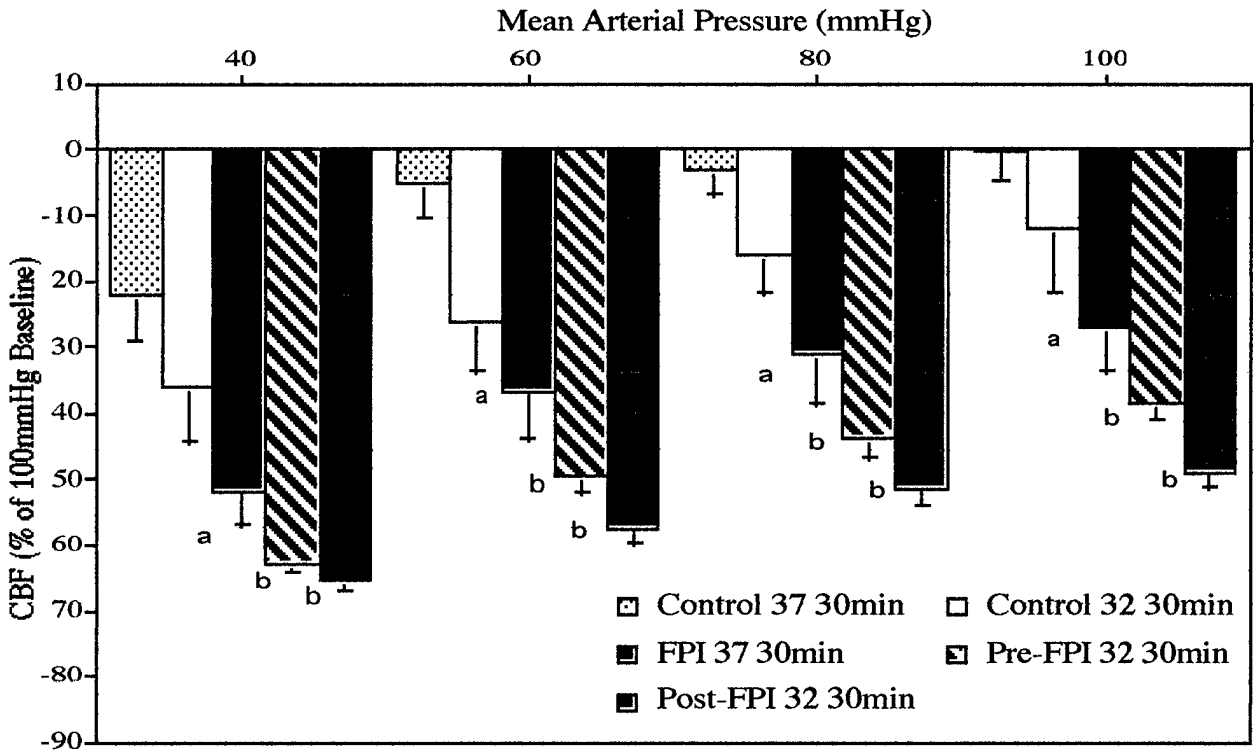


Figure 25 - Comparison of percent changes in CBF (compared to 100 mm Hg baseline) across groups 30 minutes after TBI. a = $p < 0.05$ compared to Control 37. b = $p < 0.05$ compared to Control 32.

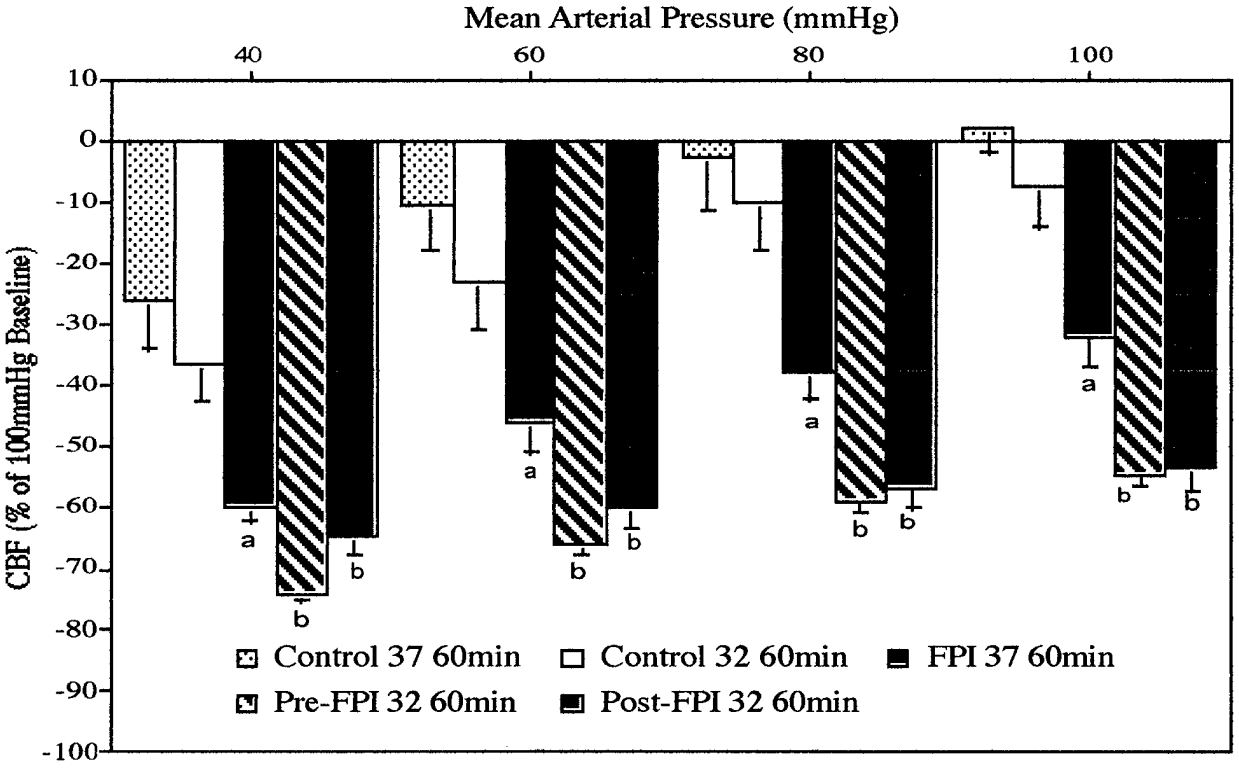
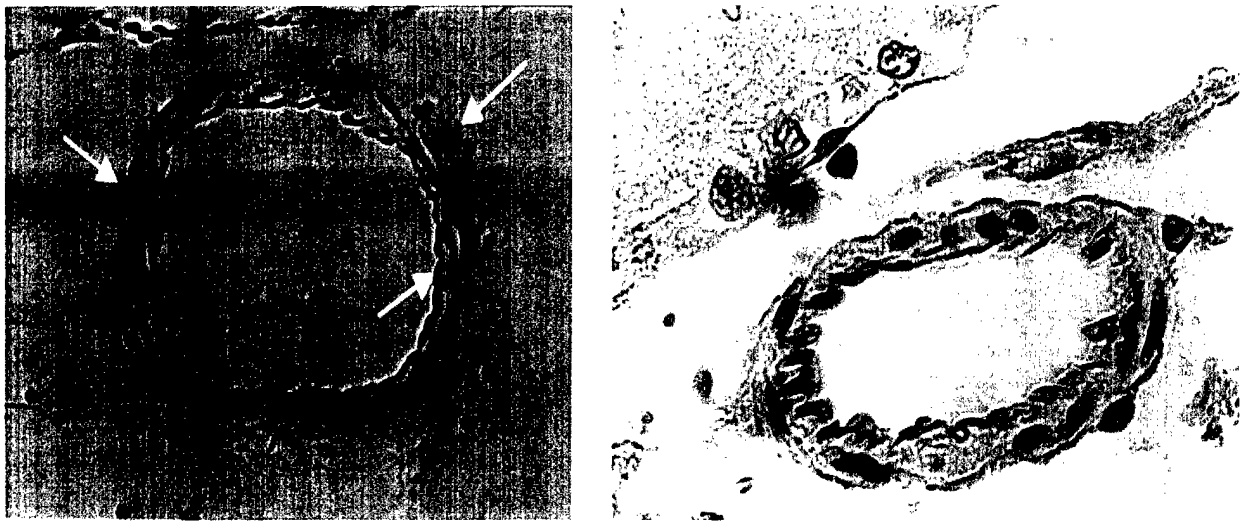


Figure 26 - Comparison of percent changes in CBF (compared to 100 mm Hg baseline) across groups 60 minutes after TBI. a = $p < 0.05$ compared to Control 37. b = $p < 0.05$ compared to Control 32.

SA 3.4 - Nitrotyrosine immunoreactivity after TBI and hemorrhagic hypotension

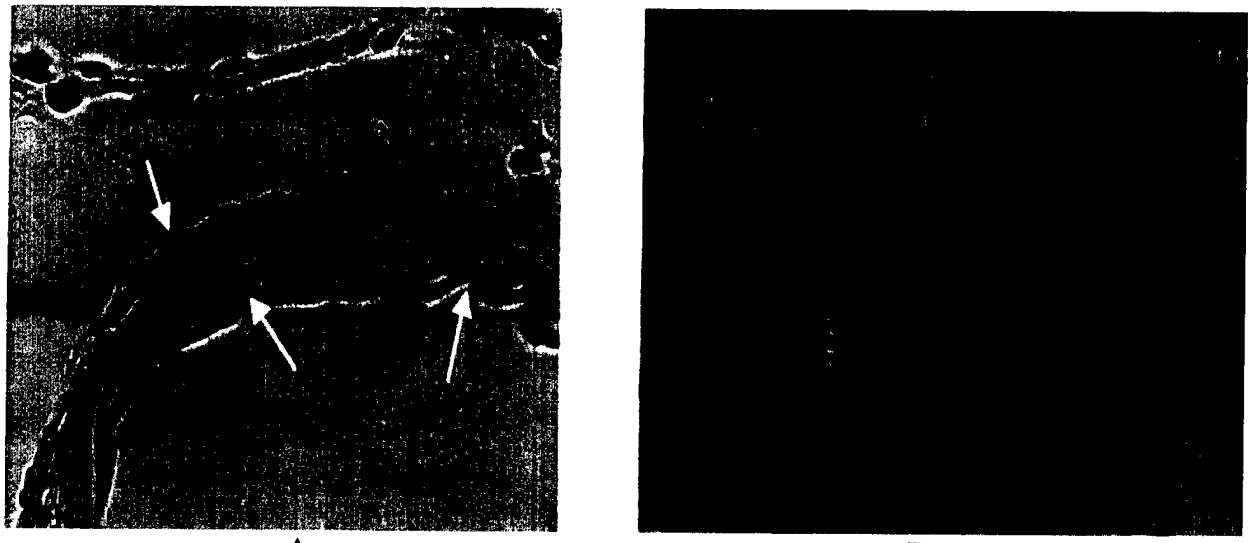
$ONOO^-$, in addition to being a powerful oxidant, is capable of nitrating (+N) or nitrosating (+NO, a.k.a. nitrosylating) phenolic compound such as tyrosine or tyrosine residues in proteins, and specific antibodies have been developed that bind with nitrotyrosine residues (25). To determine whether TBI produced nitrotyrosine and, indirectly, $ONOO^-$, specific antibodies to nitrotyrosine were used to detect nitrotyrosine in paraffin-embedded sections from rats subjected to moderate (2.0) TBI or to sham-injury. Rats were subjected to moderate TBI (n=3) or sham-injury (n=3), allowed to survive for 3 days, and then reanesthetized with isoflurane and perfused transcardially with 0.9% sodium chloride followed by 10% buffered formalin. The brains were removed, coronally sectioned, dehydrated with graded alcohols and xylene, and embedded in paraffin. 10 μM sections were cut with a rotary microtome, deparaffinized in graded alcohols and xyline, and immersed in 100% methanol and then 1% serum albumin. The sections were then incubated with a polyclonal antibody (rabbit) to nitrotyrosine overnight and rinsed and incubated with a biotinylated secondary antibody for 10 minutes, and the antigen-antibody complex was visualized using a commercially available stain. The sections were then counterstained with hematoxylin, dehydrated and coverslipped. Adjacent sections on each slide were treated identically but were not incubated with the primary antibody.

Nitrotyrosine immunoreactivity was observed in pial arteries and arterioles in the rats subjected to TBI. Immunoreactivity appeared to be mostly localized in the tunica adventitia, with some staining observed in the endothelial layer (Figure 27A & 28A). Very little nitrotyrosine immunoreactivity was observed in arterioles from the sham-injured rats (Figure 27B) and none was observed in the sections not treated with the anti-nitrotyrosine antibody (28B). These results, indicating the presence of nitrotyrosine immunoreactivity in the adventitial and endothelial layers of cerebral arteries after TBI, suggest that TBI results in the production of $ONOO^-$ within 3 days of TBI.



A. **B.**

Figure 27. Nitrotyrosine immunoreactivity (arrows, 27A) in cerebral arteries harvested from rats 3 days after moderate TBI (2.0, 27A, 400X) or sham-injury (27B, 400X).



A. B.

Figure 28. Nitrotyrosine immunoreactivity (arrows, 28A) in cerebral arteries harvested from rats 3 days after moderate TBI (2.0 atm.). Sections were treated (28A, 1000X) or not treated (28B) with a polyclonal antibody against nitrotyrosine with a hematoxyline counterstain.

SA 3.5 - Effects of peroxynitrite on myogenic responses in isolated, pressurized middle cerebral arteries

We have reported that TBI reduces CBF in rats and that L-arginine can prevent post-TBI hypoperfusion of SOD can restore CBF to baseline (8), and that superoxide anion radicals are produced by TBI (29). These results suggest that TBI may be producing superoxide radicals that destroy NO, but it is also possible that superoxide is not simply destroying NO but converting it to another vasoactive agent such as $ONOO^-$. Superoxide reacts with NO to produce the $ONOO^-$, a powerful oxidizing agent (34,35). In addition to vasoconstriction due to reductions in NO concentrations, $ONOO^-$ produced by TBI may damage the cerebral circulation and reduce compensatory responses such as autoregulation. In order to test the hypothesis that $ONOO^-$ may contribute to reduced vasodilatory responses to hypotension, MCAs were harvested from uninjured rats (n=6) and mounted on micropipettes as described above. MCA diameters were measured as intramural pressure was reduced sequentially from 100 mm Hg to 20 mm Hg in 20 mm Hg increments. Intramural pressure was then returned to 100 mm Hg and $ONOO^-$ (10-40 μM) was added to the bath. Intramural pressure was then sequentially reduced from 100 to 20 mm Hg, and arterial diameters were recorded at each 20 mm Hg increment. Transmural pressure was then returned to 100 mm Hg and MCA's diameters were measured in response to serotonin and acetylcholine. 10-40 μM $ONOO^-$ reduced myogenic vasodilation in a dose-dependent manner (Figure 29). 10 and 25 μM $ONOO^-$ had no effect on vasoconstriction to serotonin or vasodilation to ACh.

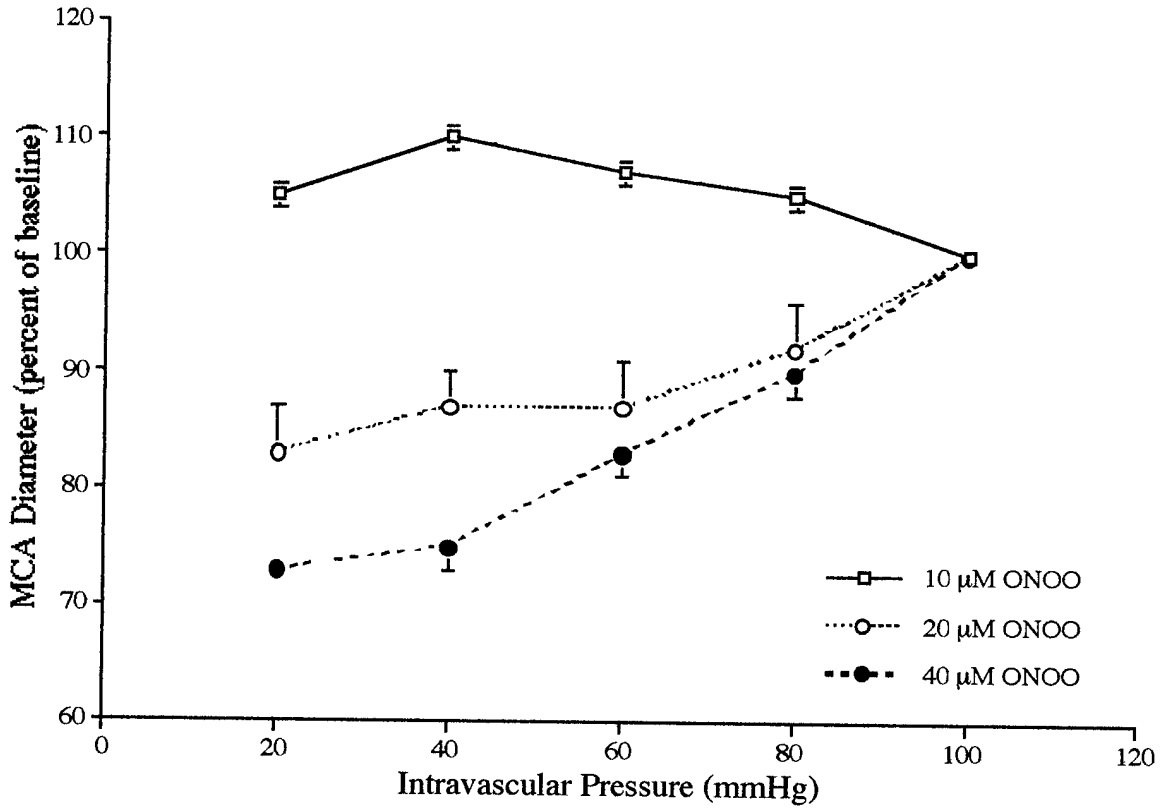


Figure 29: Inner diameters of MCAs from rats treated with 10, 25 or 40 μM peroxynitrite ($ONOO^-$).

These studies, which are the first to demonstrate that $ONOO^-$ causes a dose-dependent reduction in vasodilatory responses to progressive hypotension in isolated MCA's, suggest that TBI may impair autoregulatory vasodilatory responses via the production of $ONOO^-$ from NO and superoxide.

SA 3.6 - Effects of $ONOO^-$ or TBI on cerebral vasodilatory responses *in vitro*

CGRP has been implicated in autoregulatory responses to hypotension (10), and K^+ channels appear to contribute to cerebral vasodilatory responses to NO and hypoxia (28). TBI reduces cerebral vasodilatory responses to activators of K^+ channels in cerebral vascular smooth muscle cells (36). The four types of K^+ channels that have been described to date are ATP-sensitive, voltage-dependent, inwardly rectifying and calcium-activated (for review, see (37,38). Voltage-dependent, inwardly rectifying, and calcium-activated K^+ channels are believed to play some role in the maintenance of a resting vasodilatory "tone." ATP-sensitive K^+ channels, which are present in the cerebral circulation, don't appear to contribute to resting tone (37) but may play some role in hypercapnic cerebral vasodilation (39). It is not known which, if any, K^+ channels mediate cerebral vasodilatory responses to reductions in intravascular pressure. In order to determine whether TBI or $ONOO^-$ alter cerebral vasodilatory responses to TBI or $ONOO^-$, rats were anesthetized with isoflurane and prepared for moderate TBI (n = 6) or sham-injury (n = 6) as described above. An additional group of rats (n = 6) was anesthetized but not prepared for TBI. MCAs were harvested and mounted on micropipettes and allowed to equilibrate as described above. After the equilibration period, the $ONOO^-$ group was exposed to 25 μM $ONOO^-$ added to the arteriograph bath. MCA diameters were then measured as CGRP (10^{-9}, $5x10^{-8}$, 10^{-8}, $5x10^{-8}$M) or cromakalim ($3x10^{-8}$, $8x10^{-8}$, $3x10^{-7}$, $8x10^{-7}$M) were added.

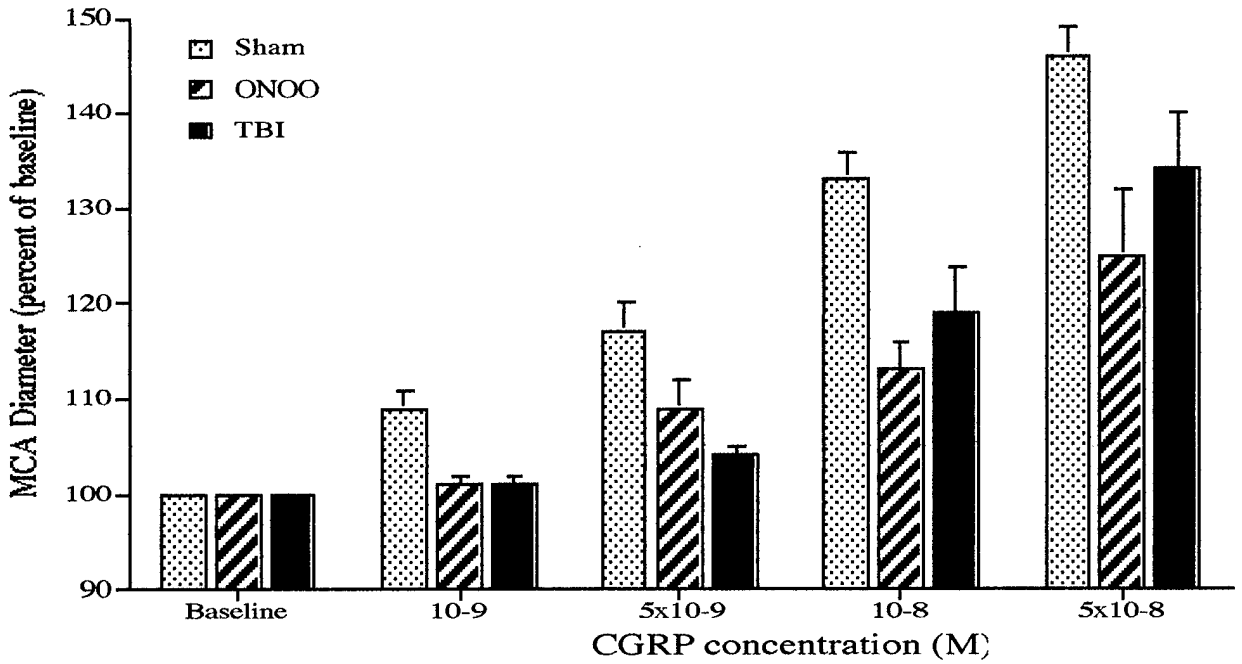


Figure 30 - MCAs diameters *in vitro* in rats treated with increasing concentrations of CGRP after moderate TBI, sham-injury, or exposure to 25μM $ONOO^-$.

In the sham-injured group, MCA diameters increased with increasing concentrations of CGRP in a concentration-dependent manner (Figure 30). In contrast, both moderate, fluid percussion TBI and 25 μM $ONOO^-$ reduced cerebral vasodilation in response to CGRP. These results suggest that TBI may impair compensatory vasodilatory responses to arterial hypotension or hypoxia by reducing vasodilatory responses to CGRP, a potent cerebral vasodilatory neurotransmitter contained in perivascular sensory nerves in the cerebral circulation. In addition, since both TBI and $ONOO^-$ reduced MCA dilation to the same degree, these data suggest that $ONOO^-$ produced by TBI may be the agent through which trauma causes cerebral vascular injury.

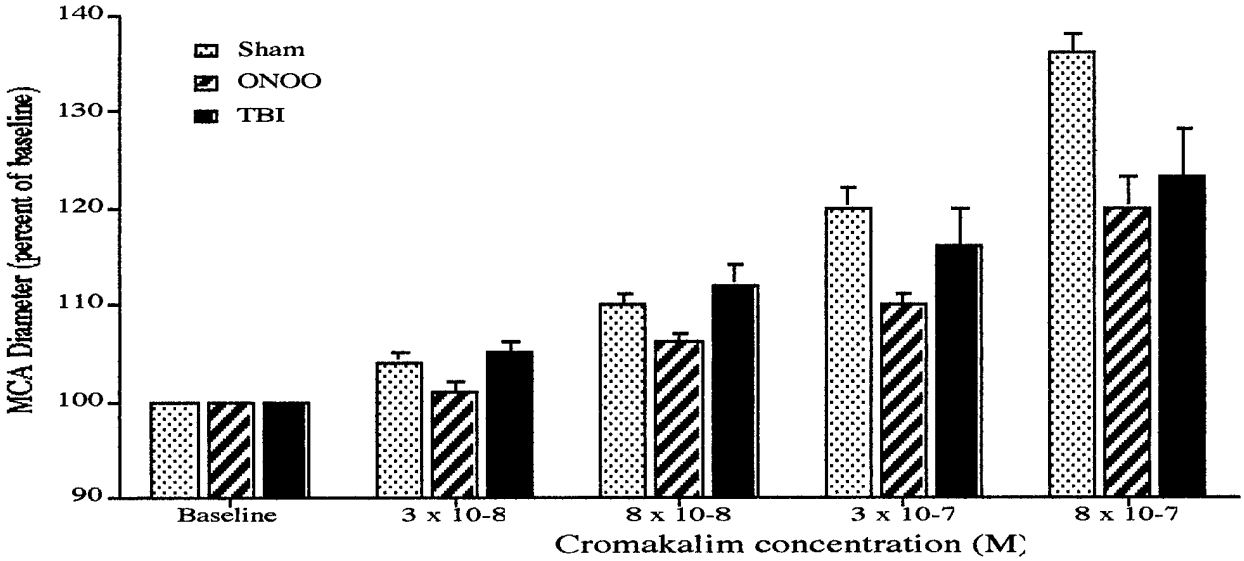


Figure 31 - MCA's diameters in vitro in rats treated with increasing concentrations of cromakalim after moderate TBI, sham-injury or exposure to 25μM $ONOO^-$.

Cromakalim caused a concentration-dependent increase in diameters in the MCAs harvested from sham-injured rats (Figure 31). Both TBI and $ONOO^-$ reduced vasodilatory responses, especially at the higher cromakalim concentrations. These results suggest that TBI reduces the vasodilatory effects of K^+ channel activation and that $ONOO^-$ plays some role in the effects of TBI on K^+ channels.

Specific Aim 4 *will address the hypothesis that small volume resuscitation with hypertonic saline will restore cerebral circulatory and systemic hemodynamics without causing the pronounced changes in brain water diffusion seen after TBI and hypotension/resuscitation with shed blood.*

The first set of experiments (SA 4.1 - Histologic effects of TBI, hypotension and resuscitation with shed blood) determine whether moderate hemorrhagic hypotension after TBI produces evidence of cerebral ischemia that was not observed in animals subjected to hypotension alone.

The second set of experiments (SA 4.2 - Effects of hypertonic solutions on CBF and ICP after TBI and hypotension) determine the effects of TBI and hemorrhagic hypotension on systemic arterial blood pressure, CBF, and intracranial pressure (ICP) after TBI, controlled hemorrhagic hypotension, and resuscitation with hypertonic, crystalloid solutions.

The third set of experiments (SA 4.3 - Effects of hypertonic resuscitation on CBF and brain edema after TBI and hypotension) tests the hypothesis that hypertonic, crystalloid resuscitation solutions improve CBF after TBI and hemorrhagic hypotension without increasing brain edema.

SA 4.1 - Histologic effects of TBI, hypotension and resuscitation with shed blood.

In order to determine the histological effects of hemorrhagic hypotension and resuscitation following TBI, rats were anesthetized with isoflurane and prepared for TBI as described above. All surgical procedures were performed aseptically, including the use of autoclaved instruments, gloves, masks, and drapes. All wound sites were scrubbed with betadine followed by an alcohol rinse prior to incision. A needle temperature probe was placed epidurally through a small burr hole lateral to the trauma device adapter ring. A cannula (PE 50) was be placed in the tail artery for monitoring arterial blood pressure. The use of the tail artery permitted measurements of arterial blood pressure without causing hindlimb ischemia, a complication which occurred occasionally when the femoral artery was used. An additional cannula (PE 90) was placed in the right jugular vein for hemorrhage and reinfusion of fluids. The rats tolerated the jugular vein cannulation better than cannulation of a femoral vein, and the jugular vein could be used for hemorrhage. The femoral vein did not permit consistent, rapid, controlled hemorrhage to 60 mm Hg. After surgical preparation, rats were randomly assigned to one of four groups:

Sham-injury - Rats were prepared for TBI but were not subjected to TBI or hemorrhagic hypotension.

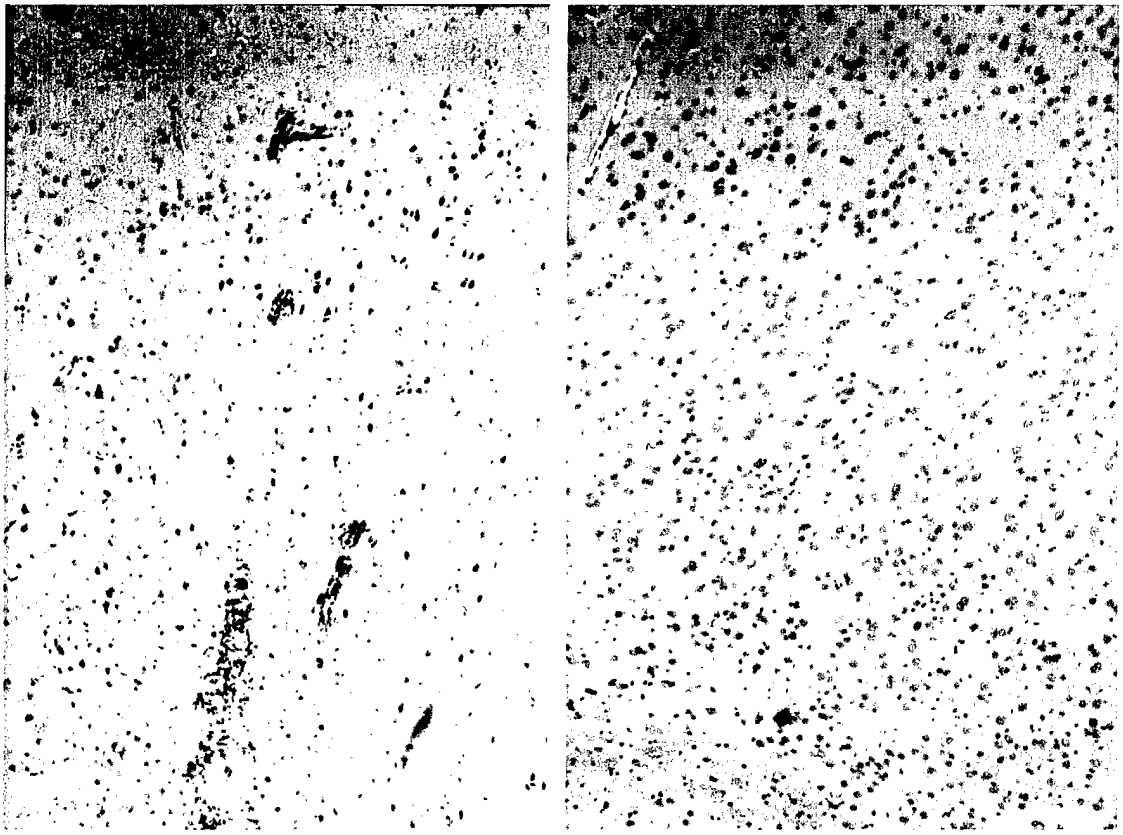
TBI-only - Rats were subjected to moderate (1.9 atm), fluid percussion TBI but were not hemorrhaged.

Hemorrhage - Rats were prepared for TBI but were subjected only to hemorrhagic hypotension (60 mm Hg for 30 min) and resuscitation with shed blood.

TBI+Hem - Rats were subjected to moderate TBI followed by hemorrhagic hypotension (60 mm Hg for 30 min) followed by resuscitation with shed blood.

After TBI and/or hemorrhage and resuscitation, wound sites were sutured, infiltrated with a long-acting topical anesthetic (bupivacaine, 0.5%) and dusted with an antibiotic powder. Rats were then treated with acetaminopen (20 mg/kg, rectal suppository) and the isoflurane was discontinued. When the rats breathed spontaneously, they were extubated and moved to a warmed, humidified chamber. Rats were observed daily and any animals exhibiting signs of infection at wound sites or of discomfort or severe neurological deficits were sacrificed

immediately. After 3 days survival, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.v.) and perfused transcardially with 10% buffered neutral formalin. Brains were stored in fixative, cut in 5mm coronal sections, dehydrated, cleared with graded alcohols, and infiltrated with paraffin. Sections were cut from coronal blocks containing the dorsal hippocampus and stained with hematoxylin and eosin. Sections were examined, and neuronal damage was evaluated and scored as the presence or absence of focal brain edema or eosinophilic neurons with nuclear degeneration.



(A) **(B)**

Figure 32 - Lateral/paramedian cortex from a rat after TBI and hemorrhagic hypotension (A) or from a rat subjected to hemorrahagic hypotension without TBI (B).

Cortical edema and shrunken, eosinophilic neurons were present in the ipsilateral hemispheres of 100% of rats subjected to both TBI and hemorrhagic hypotension (Figure 32A). No eosinophilic neurons were observed in the rats subjected to hemorrhagic hypotension alone (Figure 32B). Ischemic neurons were observed in the ipsilateral hemispheres of approximately 40% of rats subjected to TBI only. These studies demonstrate that TBI + hemorrhagic hypotension produces consistent histologic evidence of cerebral ischemia that is not present in rats subjected to hemorrhagic hypotension without TBI. These data indicate that even moderate TBI renders the brain sensitive to ischemic injury during relative mild levels of hypotension that alone produce no ischemic injury.

SA 4.2 - Effects of hypertonic solutions on CBF and ICP after TBI and hypotension

L-arginine, the precursor of the endothelium-dependent vasodilator, nitric oxide (NO), completely prevents the hypoperfusion that occurs after experimental TBI in rats (8). We have made direct simultaneous measurements of cortical blood flow and $\cdot O_2^-$ levels after TBI in rats (8,29) that suggest that increases in $\cdot O_2^-$ levels immediately after TBI initiate a process that results in hypoperfusion after TBI. We have demonstrated that treatment with the NOS substrate L-arginine prevented CBF reductions and that SOD restored CBF after TBI, suggesting that TBI-generated oxygen radicals inactivate NO or NOS and reduce CBF (8). Further studies have indicated that TBI does not affect NOS activity directly (40), suggesting that TBI directly inactivates NO rather than affecting NOS activity. What remains to be determined is whether L-arginine effectively restores CBF after TBI and hemorrhagic hypotension and which dose of L-arginine in hypertonic saline is most effective.

Rats were prepared for TBI and measurement of CBF with LDF as described above. Arterial blood pressure, ICP (Camino probe and monitor), and CBF were measured in rats treated with one of three doses of L-arginine (50, 100, or 300 mg/kg, i.v., n=6 per group) in hypertonic solutions (2400 mOsm total in NaCl) after TBI (moderate FPI) and hemorrhagic hypotension (MAP=60 mm Hg for 45 min). CBF, ICP, and MAP were monitored continuously and recorded prior to TBI (Baseline), during the hemorrhage period (Hem.), after reinfusion with one of the hypertonic arginine solutions (Inf.), and at 30, 60 and 120 min after reinfusion.

All three hypertonic arginine solutions improved MAP nearly to baseline immediately after reinfusion, but MAP fell to 59%, 68%, and 60% of baseline in the 50, 100, and 300 mg/kg L-arginine groups, respectively (Figure 33).

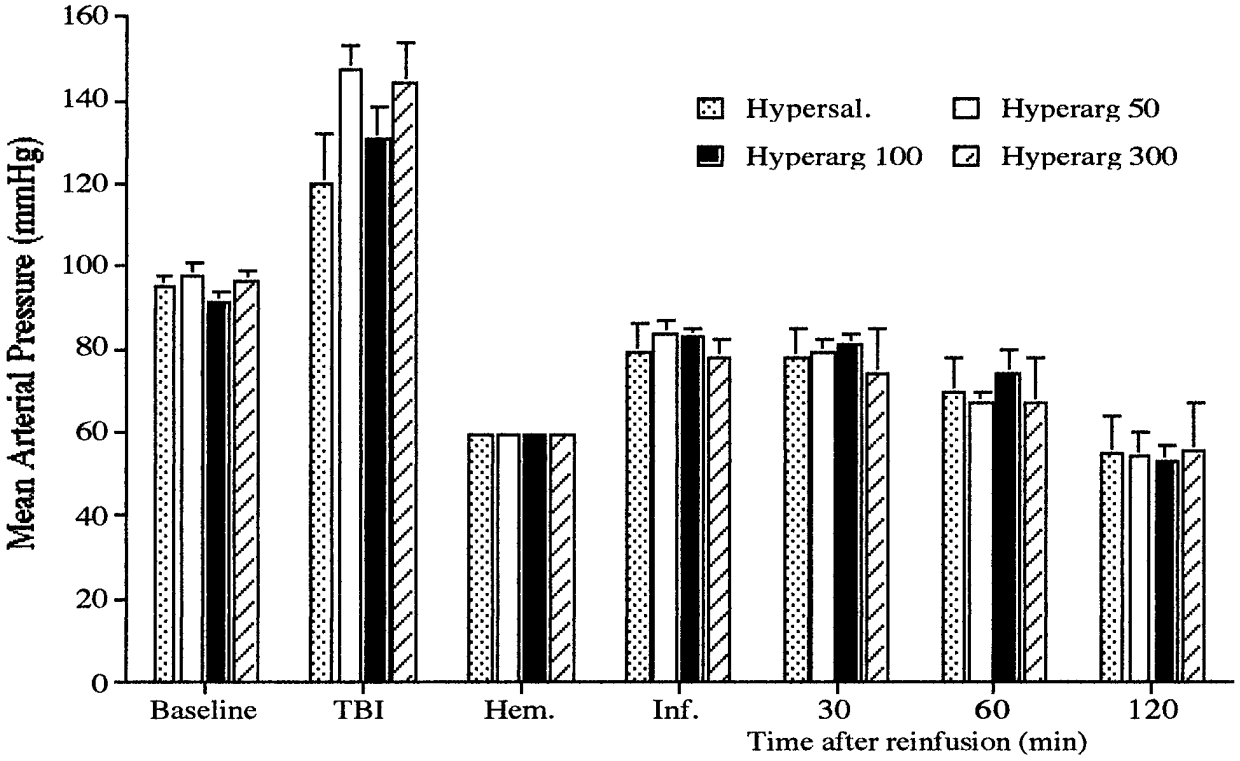


Figure 33 - MAP in rats after moderate, paramedian, fluid-percussion TBI (TBI), hemorrhage for 30 minutes (Hem.), infusion (Inf.) with 7.5% hypertonic saline (Hypersal., n = 6) or hypertonic saline with 50 (Hyperarg 50, n = 6), 100 (Hyperarg 100, n = 6), or 300 mg/kg (Hyperarg 300, n = 6), and at 30, 60 and 120 minutes after infusion. All values are mean ± standard error of the mean.

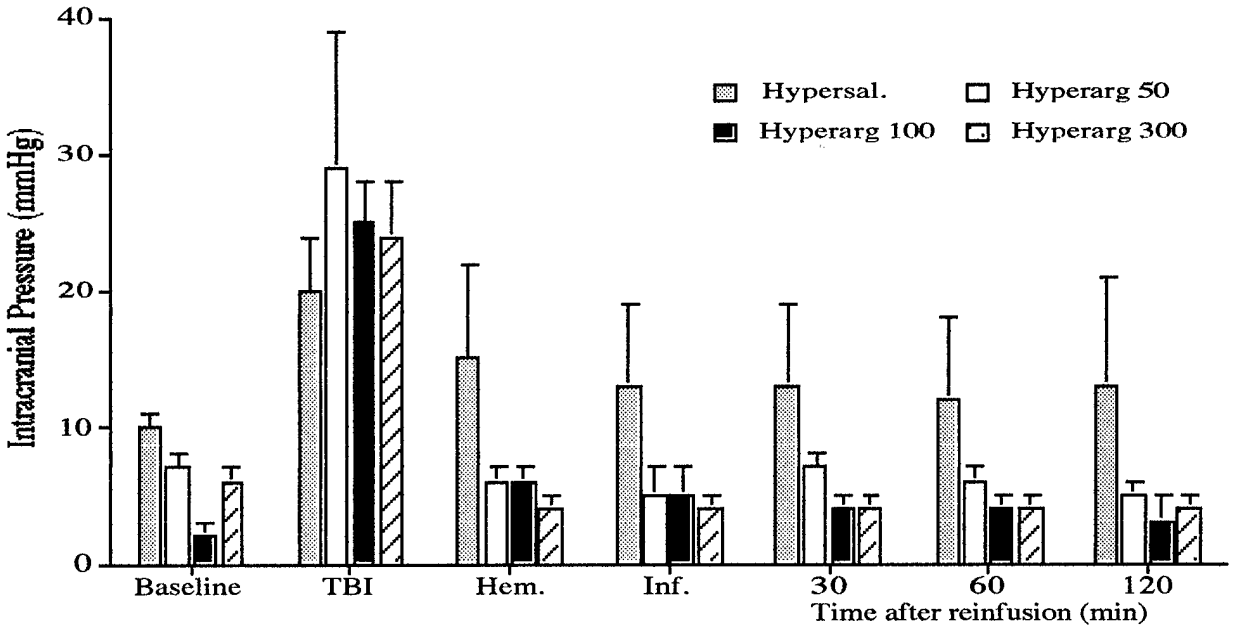


Figure 34 - ICP in rats after moderate, paramedian, fluid-percussion TBI (TBI), hemorrhage for 30 minutes (Hem.), infusion (Inf.) with 7.5% hypertonic saline (Hypersal., n = 6) or hypertonic saline with 50 (Hyperarg 50, n = 6), 100 (Hyperarg 100, n = 6), or 300 mg/kg (Hyperarg 300, n = 6), and at 30, 60 and 120 minutes after infusion. All values are mean ± standard error of the mean.

ICP increased in all groups, increasing after TBI and then decreasing during hemorrhage and resuscitation (Figure 34). ICP remained higher in the group resuscitated with hypertonic saline during hemorrhage and subsequent resuscitation.

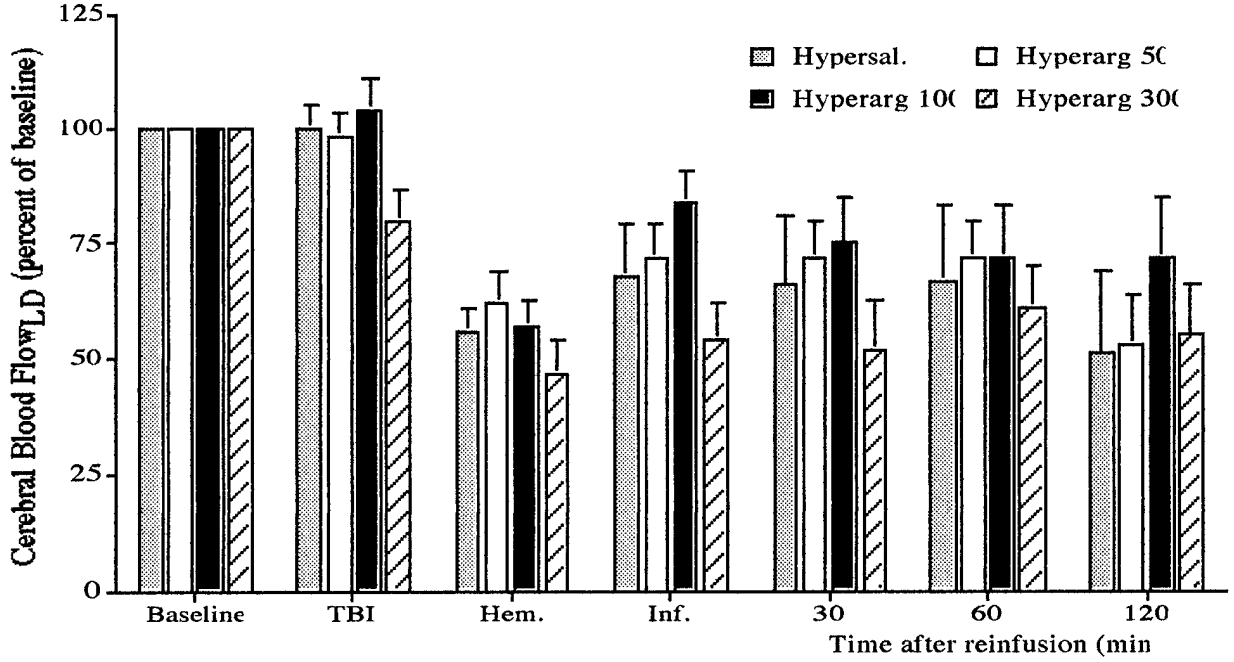


Figure 35 - Laser Doppler CBF in rats after moderate, paramedian, fluid-percussion TBI (TBI), hemorrhage for 30 minutes (Hem.), infusion (Inf.) with 7.5% hypertonic saline (Hypersal., n = 6) or hypertonic saline with 50 (Hyperarg 50, n = 6), 100 (Hyperarg 100, n = 6) or 300 mg/kg (Hyperarg 300, n = 6), and at 30, 60 and 120 minutes after infusion. All values are mean ± standard error of the mean percent of pre-TBI baseline.

CBF decreased to about 60% of baseline in all groups during hemorrhage after TBI (Figure 35). During reinfusion and 30 and 120 minutes after reinfusion, CBF levels were highest in the group treated with a hypertonic arginine solution containing 100 mg/kg of L-arginine. After reinfusion, CBF in the Hyperarg 100 group was 86% of baseline, compared to 68% and 54% of baseline in the Hyperarg 50 and Hyperarg 300 groups, respectively. Similarly, 2 hours after resuscitation, CBF in the Hyperarg 100 group was 77% of baseline, compared to 59% and 56% of baseline in the Hyperarg 50 and Hyperarg 300 groups, respectively. Therefore, these "dose-response" studies suggest that hypertonic saline-L-arginine solutions can partially restore CBF after TBI and hemorrhage and, since CBF was 77% of baseline CBF 120 minutes after reinfusion, that CBF restoration persists for hours after treatment. These studies demonstrate that the 100 mg/kg dose of arginine in hypertonic saline is the most effective concentration in terms of maximizing CBF while minimizing ICP.

SA 4.3 - Effects of hypertonic resuscitation on CBF and brain edema after TBI and hypotension

Male Sprague-Dawley rats (452 ± 10g, n=26) were anesthetized with 4% isoflurane in O_2:air (50:50), intubated, and mechanically ventilated at an isoflurane concentration of 2% in O_2:air (20:80). The rats were then prepared for TBI, hemorrhagic hypotension and resuscitation as described above (see SA 1.3 and SA 1.4). After preparation, the rats were placed in specially designed plexiglass cradles, and each cradle was placed in the magnet (Oxford Instruments, 4.7T). The coil was tuned to the proton frequency (200.056 MHz) and the magnetic field throughout the sample volume was then maximized by shimming on the water FID. Once a series of pilot images had been acquired to allow for planning of the transverse imaging slices, a high-resolution image was acquired and the FPI device was calibrated and connected to the trauma adapter. Another high-resolution image was then acquired, followed by a DWI experiment (three diffusion weighting of 0, 3 and 5 gauss/cm, tr = 3 sec, nv = 128, nt = 1) and a high-resolution reference movie (TE = 0.003 sec, tr = 0.008 sec, nv = 128, nt = 2). After placement in the magnet, rats were randomly assigned to one of the groups below:

Group One = TBI + 45 minutes hypovolemic hypotension to 60 mm Hg followed by resuscitation with shed blood.

Group Two = TBI + 45 minutes hypovolemic hypotension to 60 mm Hg followed by resuscitation with hypertonic L-arginine/saline (100 mg/kg L-arginine in 7.5% NaCl to 2400 mOsm total).

Group Three =TBI + 45 minutes hypovolemic hypotension to 60 mm Hg followed by resuscitation with hypertonic D-arginine/saline (100 mg/kg D-arginine in 7.5% NaCl to 2400 mOsm total).

Group Four = Control (sham injury)

The sequence of bolus-tracking movies, contrast injection ,and DWI acquistion were the same as described for SA 1.3 and SA 1.4. K-space filling of the bolus-tracking movie using the reference movie was performed to allow for enhanced spatial resolution. Movies will be constructed in a file format for use in an on-site developed software package (Transit) and analyzed using the following methodology. An artery and vein at the base of the ipsilateral hemisphere of the brain will be selected to provide the input and post-cerebral vascular functions respectively. From this data the passage time (To-TOA) of the bolus in seconds, as well as the total signal observed will be determined for each vessel. The majority of the parenchyma of the brain will then be outlined and the average flow curve will be determined to give the AUC and To-TOA. These data will be used to calculate the: TT (in seconds), CBV (expressed as percent of total brain volume), and CBF (expressed as ml $\cdot$100 g tissue^{-1} $\cdot$ min^{-1}).Similarly, ADC's will calculated from the experiments described above. ADCs will be calculated at baseline, immediately after TBI, at the beginning and end of the 45-minute period of hemorrhagic hypotension, and after resuscitation.

These experiments were completed in mid-December and we are currently working of data analysis.

(7) KEY RESEARCH ACCOMPLISHMENTS:

During the funding period of this grant (1 December, 1996 to 30 November, 1999), the following accomplishments were achieved:

—» We reported that L-arginine, a substrate for NO synthase, restores CBF after TBI (8). These studies provided the first evidence that NO is involved in the pathophysiology of TBI.

—» We reported that TBI does not reduce basal or stimulated hemispheric NOS activity (40). These were the first measurements of NOS activity after TBI.

—» We described the effects of changes in the cerebral vascular input functions on CBF determinations using MRI. These important studies characterized the effects of both pharmacologic (1) and physiologic and pathologic alterations (see Williams, et al., appendix) in input functions.

—» We reported that TBI does not alter basic vasodilator or vasoconstrictor mechanisms in cerebral vascular smooth muscle (41). These novel and significant observations indicated that cerebral vascular smooth muscle is fundamentally intact after TBI, which allowed investigators to focus on other aspects of the cerebral arterial wall.

—» We have observed that NOS inhibitors further reduce CBF after TBI, suggesting that TBI destroys some, but not all, of the NO that contributes to normal vasodilatory tone in the cerebral circulation.

—» We reported that myogenic vasodilatory responses to transmural pressure isolated MCA segments are significantly reduced by TBI (3). These first reports of significant impairment of cerebral vascular reactivity *in vitro* after TBI will permit electrophysiologic, biochemical, and molecular biological studies that are difficult or impossible to perform in intact animals.

—» We observed that the destruction of perivascular sensory nerves or the inhibition of cannabinoid 1 receptors reduce myogenic vasodilatory responses to reduced intravascular pressure *in vitro*. These novel results, suggesting that perivascular sensory nerves contribute to autoregulatory vasodilatory responses to hypotension through the activation of CB1 receptors, are the first such report in the cerebral circulation.

—» We reported (21) the experimental free radical scavenger and inhibitor of lipid peroxidation, U-74389G, significantly reduces $\cdot O_2^-$ production after TBI without restoring CBF, suggesting that the small increases in $\cdot O_2^-$ that we observed in the presence of U-74389G were sufficient to reduce CBF.

—» We observed that hypothermia does not improve autoregulation after TBI. These are the first data indicating that hypothermia, which is neuroprotective is models of experimental TBI, does not protect the cerebral vasculature.

—» We observed nitrotyrosine immunoreactivity in cerebral arteries after TBI and hemorrhagic hypotension, indicating that the powerful oxidant peroxynitrite ($ONOO^-$) is produced by

TBI and hypotension.

—» We reported $ONOO^-$ reduces myogenic vasodilatory responses to reduced intravascular pressure *in vitro* (27) (submitted). We also observed that $ONOO^-$ reduces vasodilatory responses to CGRP and the K^+ channel activator, cromakalim. These are the first reports of the effects of $ONOO^-$ on vasodilatory responses in the cerebral circulation.

—» We observed that TBI and hemorrhagic hypotension produces cerebral ischemia that is not observed with the same level of hypotension in the absence of TBI. We believe that these are the first successful survival studies of the effects of hemorrhagic hypotension and resuscitation after TBI.

(8A) REPORTABLE OUTCOMES:

The following articles, book chapters, editorials, and abstracts were published or submitted during the previous funding period. Items included in Appendix 1 are marked with an asterisk.

*1. Mottet, I, Quast MJ, DeWitt DS, Hillman GR, Wei J, Uhrbrock DH, Perez-Polo JR, Kent TA. Nw-nitro-L-arginine methyl ester modifies the input function measured by dynamic susceptibility contrast MRI. J Cereb Blood Flow Metab 17:791-800, 1997

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34. Bedell EA, DeWitt DS, Prough DS. Comparison of cerebral blood flow (CBF) measurements by hydrogen clearance and laser Doppler flowmetry (LDF) during progressive hemodilution in rats. Anesthesiology 90:A728, 1999 (abstract)

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*39. DeWitt DS, Mathew B, Chaisson JM, Prough DS. Peroxynitrite reduces vasodilatory

responses to progressive reductions in intravascular pressure in isolated middle cerebral arteries. Stroke (in review).

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(8B) PERSONNEL:

NAME	EFFORT	ROLE
Douglas S. DeWitt, Ph.D.	40%	Principle Investigator
Donald S. Prough, M.D.	5%	Co-Investigator
Thomas A. Kent, M.D.	20%	Co-Investigator
Michael J. Quast, Ph.D.	10%	Co-Investigator
Kimberly Miller, B.S.*	100%	Research Assistant
Jennifer M. Chaisson, B.A.*	100%	Research Assistant

* During the funding period, Ms. Miller quit to attend medical school and was replaced by Ms. Chaisson.

(9) CONCLUSIONS:

Successful treatment of patients, both military and civilian, after TBI and hemorrhagic hypotension requires a thorough understanding of the mechanisms that contribute to trauma-induced cerebral vascular dysfunction. During the course of the funding period of this application, we made novel and important contributions to a better understanding of the pathophysiology of traumatic vascular injury.

We reported a model that allows for sequential, accurate and quantitative measurements of regional CBF using MRI. We have used MRI to measure CBV, TT, CBF, and brain and arterial AUC after TBI only, hemorrhagic hypotension only, or combined injury. These are the only quantitative measurements of these parameters that have been made in animal models of TBI or TBI and hypotension.

We reported that L-arginine (100 mg/kg) prevented CBF reductions after TBI. These studies provided the first evidence that NO was involved in cerebral vascular dysfunction after TBI. These observations led us to combine L-arginine with hypertonic (7.5%) saline in a blood replacement fluid for which we have applied for a United States patent. We observed that 100 mg/kg hypertonic L-arginine improves CBF more effectively than 50 mg/kg or 300 mg/kg hypertonic L-arginine without increasing ICP. Although further histologic and behavioral studies of hypertonic L-arginine are required, the formulation could prove to be an effective blood replacement product.

Our studies of the effects of TBI on isolated, pressurized MCA segments have yielded several important observations about the mechanisms that contribute to traumatic vascular dysfunction. We are the first to report that TBI doesn't alter basic vasodilator or vasoconstrictor mechanisms in cerebral vascular smooth muscle (41). We reported that myogenic vasodilatory responses to transmural pressure isolated MCA segments are significantly reduced by TBI (3). These first reports of significant impairment of cerebral vascular reactivity *in vitro* after TBI will permit electrophysiologic, biochemical and molecular biological studies that are difficult or impossible to perform in intact animals.

Using the *in vitro* MCA preparation, we demonstrated that $ONOO^-$ can abolish

compensatory vasodilatory responses to hypotension in isolated MCAs. We also observed that $ONOO^-$ reduces vasodilatory responses to CGRP and the K^+ channel activator, cromakalim. These are the first reports of the effects of $ONOO^-$ on vasodilatory responses in the cerebral circulation. We observed nitrotyrosine immunoreactivity after TBI and hemorrhagic hypotension, indicating the interaction between $ONOO^-$ and tyrosine residues in proteins. Together, these observations indicate that $ONOO^-$ is produced by TBI and hypotension, that $ONOO^-$ is present in sufficient quantities to attack cellular proteins, and that $ONOO^-$ is capable of significantly reducing cerebral vasodilatory responses. This evidence that $ONOO^-$ contributes to the pathophysiology of traumatic vascular injury should lead to novel therapeutic strategies as specific inhibitors of $ONOO^-$ are developed.

We have reported novel and exciting evidence phenol, which destroys perivascular sensory nerves, and SR141716A, a specific antagonist of CB1 receptors, reduce myogenic responses to hypotension. These novel results, suggesting that perivascular sensory nerves contribute to autoregulatory vasodilatory responses to hypotension through the activation of CB1 receptors, are the first such report in the cerebral circulation. Although further studies are necessary to test these hypotheses, our data suggest that TBI may impair the CB1 receptor or may reduce the levels or production of anandamide by the brain or by perivascular nerves. These observations suggest that an untested class of compounds, those that act at cannabinoid receptors, may improve cerebral vascular function after TBI.

We have completed a series of TBI and hemorrhagic hypotension survival studies which we believe are the first successful survival studies of the effects of hemorrhagic hypotension and resuscitation after TBI. Our studies demonstrated that the combined insult produced ischemic neuronal injury in 100% of animals studied while there was no evidence of ischemia in rats subjected to hemorrhage only. This model of ischemic neuronal injury and survival after TBI and hypotension will allow us to test the effects of treatments that may improve cerebral vascular function on neuronal survival.

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(11) APPENDIX

The publications and manuscripted listed below are included in the Appendix:

1. Mottet, I, Quast MJ, DeWitt DS, Hillman GR, Wei J, Uhrbrock DH, Perez-Polo JR, Kent TA. N^{w}-nitro-L-arginine methyl ester modifies the input function measured by dynamic susceptibility contrast MRI. J Cereb Blood Flow Metab 17:791-800, 1997

2. DeWitt DS, Smith TG, Deyo DS, Miller KR, Uchida T, Prough DS. L-arginine or superoxide dismutase prevents or reverses cerebral hypoperfusion after fluid-percussion traumatic brain injury. J Neurotrauma,14:223-233, 1997

3. Bukoski RD, Wang SN, Bian K, DeWitt DS. Traumatic brain injury does not alter cerebral contractility. Am J Physiol 272:H1406-H1411, 1997

4. Alagarsamy S, DeWitt DS, Johnson KM. Traumatic brain injury does not affect nitric oxide synthase activity in rats. J Neurotrauma 15: 625-631, 1998.

5. Mathew B, DeWitt DS, Bryan RM, Bukoski RD, Prough DS. Traumatic brain injury reduces myogenic responses in pressurized rodent middle cerebral arteries. J Neurotrauma 16:(in press), 1999

6. DeWitt DS, Mathew B, Chaisson JM, Prough DS. Peroxynitrite reduces vasodilatory responses to progressive reductions in intravascular pressure in isolated middle cerebral arteries. Stroke (in review).

7. DeWitt DS, Prough DS, Deal DD, Vines SM, Uchida T. Inhibition of nitric oxide synthase does not reduce cerebral vascular responses to hemodilution or hemorrhagic hypotension in rats. J Neurosurg Anesth (in review).

8. DeWitt DS, Mathew B, Prough DS, Uchida T, Bian K, Bukoski RD. Modulation of myogenic tonic by perivascular sensory nerves. Am J Physiol (in review).

9. Williams JP, DeWitt DS, Quast MJ, Kent TA, Prough DS. Effects of Fluid Percussion Brain Injury and Mild Hemorrhagic Hypotension on Cerebral Blood Flow: an MRI study. J. Neurotrauma (in review).

Journal of Cerebral Blood Flow and Metabolism
17:791–800 © 1997 The International Society of Cerebral Blood Flow and Metabolism
Published by Lippincott-Raven Publishers, Philadelphia

N^G-Nitro-L-Arginine Methyl Ester Modifies the Input Function Measured by Dynamic Susceptibility Contrast Magnetic Resonance Imaging

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Summary: In rat brain dynamic susceptibility contrast magnetic resonance (MR) images, vessels visible on the same scan plane as the brain tissue were used to measure the characteristics of the input function of the MR contrast agent gadopentetate dimeglumine. MR images were acquired 30 and 60 minutes after intravenous injections of 3 mg/kg and 15 mg/kg N^G-Nitro-L-arginine methyl ester (L-NAME) (n = 9). The time of arrival (TOA) and the mean transit time corrected for TOA of the input function were increased by 3 mg/kg or 15 mg/kg L-NAME. The area of the input function was increased by 15 mg/kg L-NAME. In two animals, similar modifications of the input function induced by 20 mg/kg L-NAME were reversed by infusion of sodium nitroprusside. In two other animals, MABP was increased by phenylephrine to a similar extent as in L-NAME experiments, but did not induce the same modifications of the input function, showing that the action of L-NAME on the input function was not simply caused by an effect on MABP. These results show that the input function can be significantly altered by manipulations widely used in cerebrovascular studies. These input function changes have important implications for calculation of cerebral blood flow. **Key Words:** Magnetic resonance imaging—Blood flow— Dynamics—brain perfusion—L-NAME.

Since the first descriptions of techniques allowing the assessment of hemodynamic parameters by using noninvasive T2*-sensitive nuclear magnetic resonance (NMR) bolus track measurements (Villringer et al., 1988; Majumdar et al., 1988; Belliveau et al., 1990; Moseley et al., 1991), this method has found widespread applications in the evaluation of neurological diseases and cerebrovascular perturbations. While passing through the microvascular bed, a bolus of paramagnetic contrast material such as gadopentetate dimeglumine alters the magnetic susceptibility of tissues and produces local field inhomogeneities, leading to reductions in T2 and T2* of the tissue. This results in transiently decreased signal intensities from T2*-weighted pulse sequences such as gradient-echoes. Acquiring a series of rapid T2*-weighted gradient-echo images during the first pass of the contrast agent bolus allows the reconstruction of a signal intensity-time curve that can be later converted into concentration-time curves. Using the indicator dilution theory, the concentration-time data can be used to calculate blood volumes and flows. In conditions where the blood-brain barrier is not impaired, gadopentetate dimeglumine remains intravascular in the brain, and the indicator dilution theory for intravascular tracers can be applied.

However, for calculation of CBF, this theory requires knowledge of the concentration-time curve of the tracer corresponding to the input of the observed tissue: the input function (Axel, 1980). Most of the previous magnetic resonance imaging (MRI) perfusion studies by dynamic susceptibility contrast experiments were per-

Received July 5, 1996; final revision received March 12, 1997; accepted March 12, 1997.

This work was funded by National Institutes of Health grants IRO1NS33288-01 and IRO1NS33575-01. Dr I. Mottet was supported by a grant from the Faculty of Medicine, Université Catholique de Louvain, Belgium.

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Abbreviations used: CBV, cerebral blood volume; CVR, cerebral vascular resistance; L-NAME, N_ω-Nitro-L-arginine methyl ester; MRI, magnetic resonance imaging; SNP, sodium nitroprusside; TOA, time of arrival.

formed in conditions where a constant input function could be assumed, such as the comparison of different areas of the same brain, allowing the calculation of relative values of cerebral blood volume (CBV) and CBF without measuring the input function.

The purpose of the present study was to determine whether changing physiological conditions would change the input function. In rat brain MR perfusion images, arteries and veins visible in the same scan plane as the brain tissue were used to measure the parameters of the input function. The physiological conditions were changed by injecting the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), already known to modify the resistance of cerebral arteries and CBF (Prado et al., 1992; Pelligrino et al., 1993). In two rats, the nitric oxide donor sodium nitroprusside (SNP) was given to reverse the modifications of the input function induced by 20 mg/kg L-NAME (Moncada et al., 1991; Smith et al., 1995). In two other rats, the MABP was increased by phenylephrine infusion to a similar extent as the hypertension induced by L-NAME injections, and the modifications of the input function compared with those of the L-NAME group. The implications of these input function changes were illustrated by evaluating CBV and CBF changes in the different conditions.

PRINCIPLES OF FLOW MEASUREMENT BY THE INDICATOR DILUTION THEORY

The indicator-dilution method using intravascular tracers converts the measurement of indicator concentration distal to an organ into flow and volume information about this organ (Zierler, 1965). This theory has been extended to the calculation of cerebral perfusion based on rapid sequence tomographies performed simultaneously with the bolus injection of a nondiffusible indicator (Axel, 1980): The Central Volume Theorem states that tissue blood flow (F) can be determined by the ratio

$$F = \frac{V}{MTT} \quad (1)$$

where V is the volume of distribution of the agent within the tissue (for an intravascular contrast agent, V is the tissue blood volume), and MTT is the mean transit time, or normalized first moment, of the outflow concentration-time curve $C_{out}(t)$ resulting from a bolus injection.

$$MTT = \frac{\int_0^\infty t\, C_{out}(t)dt}{\int_0^\infty C_{out}(t)dt} \quad (2)$$

Assuming that the concentration of the contrast material is the same at both points, $C_{out}(t)$ may be measured either at the outflow orifice (the vein) or within the central volume (the tissue level) (Axel, 1980). This assumption justifies the use of the first moment of C(t) measured in the tissue in most of the MR perfusion studies, but the accuracy of this approach has already been questioned (Hamberg et al., 1993; Weisskoff et al., 1993).

The definition of MTT involves the transit of an idealized narrow square-wave bolus from an instantaneous delta function injection. Practically, however, the intravenous injection of the MR contrast agent has finite duration and furthermore will be delayed and prolonged before reaching the artery supplying the observed tissue. Consequently, the actual observed tissue function $C_{obs}(t)$ is the convolution of the input function $C_a(t)$ and the residue function $C_{tissue}(t)$, the tissue concentration-time curve that would have been achieved with an ideal instantaneous injection.

$$C_{obs}(t) = C_a(t) * C_{tissue}(t)$$

To find $C_{tissue}(t)$, $C_{obs}(t)$ should be deconvolved with $C_a(t)$. However, the first moments of convolved functions are generally additive (Zierler, 1962), so that

$$MTT_{obs} = MTT_a + MTT_{tissue}$$

which gives a simpler method for correcting MTT, as long as $C_a(t)$ and its associated MTT_a can be measured. This can be done from the MR signal of an artery feeding the organ and appearing in the scan plane.

Using an intravascular contrast agent, MTT_{tissue} only defines the flow per unit vascular volume. To calculate the flow per unit tissue volume, the fraction of vascular volume in the tissue (CBV for cerebral perfusion studies) must be determined. The absolute value of CBV can be calculated by integrating the observed tissue concentration-time curve and normalizing to the integrated arterial input function, or to the integrated draining vein function $C_v(t)$, as it can be shown that $\int C_a(t)dt = \int C_v(t)dt$ (Axel, 1980). Thus

$$CBV = \frac{\int_0^\infty C_{obs}(t)dt}{\int_0^\infty C_{alv}(t)dt} \quad (3)$$

$C_v(t)$ can be measured in a venous structure appearing in the scan plane, such as the superior sagittal sinus. Without knowing the area under $C_a(t)$ or $C_v(t)$, a constant value may be assumed and relative values of CBV may be estimated based on the measurement of $\int C_{obs}(t)dt$ alone. For comparisons of CBV measured under different physiological situations, the assumption of a constant input function may not always hold. Similarly, the knowledge of $C_a(t)$ is required for the calculation of an absolute value of the tissue flow, as MTT_{obs} (in the venous output or in the tissue itself as discussed above) must be corrected by MTT_a. Other models incorporating

the input function have been proposed (Larson, et al., 1994).

MATERIALS AND METHODS

Animal model

All animal procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee. Male Sprague-Dawley rats (310 to 490 g body weight) were initially anesthetized with 4% halothane in balanced breathing air (30% O_2/70% N_2). Rats were then intubated and mechanically ventilated using a modified compressed air-powered clinical pressure ventilator (Monaghan, Littleton, CO, U.S.A.), with halothane maintained at 0.6 to 1% during the surgery and MRI procedures. A femoral arterial cannula was inserted for blood gas determination and MABP monitoring. A cannula was inserted in the femoral vein to deliver the bolus of gadopentetate dimeglumine (Magnevist, Berlex Imaging, Wayne, NJ, U.S.A.), the intravascular MR contrast agent, and of N^G-Nitro-L-arginine methyl ester (L-NAME). A second intravenous femoral cannula was inserted when necessary for SNP (Elkins-Sinn, Cherry Hill, NJ. U.S.A.) or phenylephrine (Elkins-Sinn) continuous infusion. The rectal temperature was kept at 37°C with a warm-water blanket during surgery. During MRI, the rat temperature was maintained by circulating warm air through the magnet bore.

Total preparation time was about 2 hours. Then two perfusion measurements by bolus-tracking T2*-weighted sequences were performed at 30-minute intervals under control conditions. Considering the stability and reproducibility of the dynamic susceptibility contrast MRI method for sequential perfusion measurements, previous investigations showed a persistent modification of the signal intensity after a second injection of gadoteridol (ProHance, Bracco Diagnostics, Princeton, NJ, U.S.A.) (Levin et al., 1995). Consequently, only the second perfusion measurement performed under control conditions was used for comparison with the measurements performed after drug administration.

In a first group of rats (L-NAME, n = 9), a first intravenous bolus injection of 3 mg/kg L-NAME was given, followed after 1 hour by a second intravenous injection of 15 mg/kg L-NAME. Perfusion measurements were performed 30 and 60 minutes after each L-NAME injection. For L-NAME + SNP experiments, two rats underwent a perfusion measurement 30 minutes after a 20 mg/kg L-NAME injection. This dose was selected because reversal of CBF effect of 20 mg/kg L-NAME by SNP has been reported (Smith et al., 1995). Then a continuous infusion of SNP (0.1 mg/mL, 20 to 40 μL/kg/min) was started at the lower dose and gradually increased until MABP was restored to its pre- L-NAME value. The infusion was then continued at this rate. A perfusion measurement was performed after a stable MAP was achieved. For the phenylephrine experiments (n = 2), phenylephrine infusion (0.25 mg/mL, 5 to 50 μL/kg/min) was started after the control perfusion measurements. Once a new steady state at high MABP was achieved, a perfusion measurement was performed. Then the phenylephrine infusion was discontinued resulting in a decrease in MABP. We waited 30 minutes between measurements.

At the end of the experimental protocol, the rats were killed with an intravenous injection of 60 mg pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.).

Magnetic resonance imaging experiments

The proton MRI experiments were performed using a 4.7 Tesla, 33-cm horizontal bore magnet (SISCO/Varian, Palo Alto, U.S.A.). Excitation and signal detection were achieved with a 6-cm surface coil. Twelve contiguous spin-echo T2-weighted coronal images were acquired, with a 2-second repetition time, 65-millisecond echotime, 128 phase-encoding steps, 4-cm field of view, and 1.7-mm slice thickness. In this set of images, the coronal plane corresponding approximately to stereotaxic coordinates +7.6 mm interaural and −1.4 mm bregma, Paxinos coordinates (Paxinos and Watson, 1986) was chosen for the single-slice bolus tracking experiments. A T2*-sensitive fast low-angle shot pulse sequence (Frahm et al., 1987) was used, with 11- millisecond repetition time, 2.5 millisecond echotime, 8-cm field of view, and 1.9-mm slice thickness. Thirty T2*-weighted images were recorded consecutively. For each perfusion measurement, a first reference movie of 30 T2*-weighted images with a matrix 128 × 128 and 2 averaged acquisitions was performed. Then a second movie was acquired with 30 T2*-weighted images with a matrix of 128 × 64 and 1 acquisition, resulting in a recording time of 0.7 seconds per image. During this second acquisition, a bolus of 0.3 mmol/kg of gadopentetate dimeglumine was injected into the femoral vein synchronously with the start of the 6th image, over a 1.5 second interval. A reconstruction algorithm used the higher phase-encoding steps of the high spatial resolution reference movie and the lower phase-encoding steps of the low spatial but high temporal resolution bolus-tracking movie to yield a 128 × 128 matrix image where the signal intensities corresponded to the bolus-tracking movie.

Data analysis

The time-course of the signal intensity was calculated for each pixel. Signal intensities were transformed into relative concentrations C(t) of the tracer (Belliveau et al., 1990).

$$C(t) = \frac{-1}{TE\, k_1} \ln \frac{S(t)}{S_0} \quad (4)$$

where TE is the echotime, S_0 is the baseline signal intensity, S(t) is the time-dependent signal intensity, and k_1 is a proportionality factor related to tissue, pulse sequence, and field strength characteristics (Quast et al. 1993).

$C_b(t)$ was determined for a region of interest covering the whole brain tissue, excluding the sagittal sinus and the basal area displaying the large vessels. To measure $C_a(t)$, a single pixel was chosen in an area corresponding to a transversally cut artery in the circle of Willis. Compared to the brain tissue, the arteries were characterized in T2*-weighted images by a higher baseline signal intensity, and an earlier decrease and recovery of the signal intensity with the passage of the contrast agent. To minimize the partial volume effect, the arterial pixel with the highest intensity achieved was chosen. $C_v(t)$ was measured in the superior sagittal sinus both in a single pixel chosen for its highest value of $\int C_v(t)dt$, and in a region of interest covering the whole area of the superior sagittal sinus.

Concentration-time curves from dynamic-susceptibility contrast MR images are usually fitted to a gamma variate model. In the present study, the data did not conform readily to this model, especially after L-NAME administration, which deformed the curve, and, in the sagittal vein, showed multiple compartments. For this reason, numerical integration was used. $C_b(t)$, $C_a(t)$, and $C_v(t)$ were integrated between the time of arrival (TOA) of the contrast agent, defined as the initial point of significant deflection from baseline, and the end of the first pass of the contrast agent, the signal corresponding to recirculation being cut off. This calculation yielded the values of $\int C_b(t)dt$, $\int C_a(t)dt$, and $\int C_v(t)dt$. For the calculation of MTT by Equation 2, t = 0 was set at the time of injection of the contrast agent. To better characterize the shape of the concentration-

time curves, the MTT corrected for TOA was also calculated, and called MTTsh. For the venous curve, MTT_v, TOA_v, and $MTTsh_v$ were calculated with the single pixel measurement of $C_v(t)$, which minimized the partial volume effect, but $\int C_v(t)dt$ was calculated from a combined group of several pixels within the superior sagittal sinus, as this value of the integral was more reproducible than in a single pixel.

Although the purpose of this experiment was to assess modifications of the input function, calculations of CBV and CBF were attempted. Cerebral blood volume was calculated according to Equation 4, by the ratio of $\int C_b(t)dt$ to $\int C_v(t)dt$ (preferred to $\int C_a(t)dt$ whose variability was higher because of the single pixel measurement of $C_a(t)$). The results (mean and SD) were converted to percents of the mean value at baseline. MTT_{tissue} was corrected for the input function according to Equation 3, by subtracting MTT_a from MTT_v, the MTT of the outflow concentration-time curve. Cerebral blood flow (CBF) was calculated by the ratio of CBV to MTT_{tissue}. Because of limited temporal resolution, the variations of MTT_{tissue} were large compared with the small absolute values of this parameter used as a denominator: therefore, the actual calculation of individual CBF values was subjected to large errors. However, CBF could be evaluated as the ratio of the mean values of CBV and MTT_{tissue}. The SD of those ratios were calculated according to Raj (1968). The absolute values of the ratios and SD were converted to percents of the ratio calculated at baseline.

Data are expressed as mean ± SD. Statistical differences were obtained using a repeated measurement analysis of variance, followed by a Bonferroni t-test for multiple group comparisons, using the statistical software SigmaStat 1.0 (Jandel, San Rafael, CA, U.S.A.). A value of $P < 0.05$ was considered to be significant for drug effects.

RESULTS

Blood gas parameters did not change immediately before and after MR imaging experiments in the L-NAME (n = 9), L-NAME + SNP (n = 2), and phenylephrine (n = 2) groups. Those parameters remained in the physiological range for rats during the whole observation period (Baker et al., 1979). L-NAME injection induced a significant increase of MABP (Table 1). The MABP for the SNP and phenylephrine-treated rats are also shown.

Representative images out of a sequence of 30 dynamic susceptibility contrast images acquired during baseline conditions are displayed in Fig. 1 for illustration of the time-course of signal intensity in arteries, brain tissue, and sagittal sinus. Signal intensities were converted to concentration-time curves (C(t)) of the contrast agent.

The effect of 3 mg/kg and 15 mg/kg L-NAME on perfusion parameters are presented in Fig. 2. After injection of L-NAME, MTT_a increased significantly, reflecting an augmentation of both TOA_a (significant 30 minutes after the low dose, and 30 and 60 minutes after the high dose of L-NAME), and $MTTsh_a$ (significant only after the high dose). $\int C_a(t)dt$ did not change significantly, although a trend toward an increase was suggested. The arterial curve seemed especially affected by limited spatial resolution. An increase of MTT, TOA, and MTTsh was observed after L-NAME in the venous and the brain functions, reaching statistical significance after 15 mg/kg L-NAME, except that TOA_v already significantly increased 30 minutes after the low dose, and $MTTsh_v$ did not reach statistical significance. Unlike $\int C_a(t)dt$, $\int C_v(t)dt$ increased significantly after 15 mg/kg L-NAME, but the increase of $\int C_b(t)dt$ was not significant.

In the two animals tested, SNP infusion reversed the effects of 20 mg/kg L-NAME on MTT, TOA, and MTTsh of the arterial, brain, and vein functions (Fig. 3). Sodium nitroprusside also reversed the increase of $\int C_v(t)dt$ induced by L-NAME. In the two animals studied, MTT, TOA, and MTTsh did not increase, or even decreased during phenylephrine infusion in the arterial, brain, and vein functions (Fig. 4). Compared to L-NAME, phenylephrine had also an opposite action on $\int C_v(t)dt$, which decreased. Stopping phenylephrine infusion reversed these parameters.

The purpose of this study was to illustrate that the input function can be affected by changes in systemic physiological conditions. However, we did preliminary calculations of CBV and CBF with and without consideration of the input function and evaluated the direction of effects. Cerebral blood volume values were calculated by Equation 4. The results are presented as percent of the baseline value. Cerebral blood volume tended to de-

TABLE 1. *Mean arterial blood pressure*

L-NAME (n = 9)		L-NAME + SNP (n = 2)			Phe (n = 2)		
	Mean ± SD		Rat 1	Rat 2		Rat 1	Rat 2
MABP (mm Hg)							
Baseline	89 ± 7	Baseline	79	85	Baseline	89	98
30 min after 3 mg/kg L-NAME	117 ± 7*	30 min after 20 mg/kg L-NAME	118	128	Phe	130	142
1 h after 3 mg/kg L-NAME	115 ± 12*	L-NAME + SNP	84	105	Discontinuation of Phe	82	100
30 min after 15 mg/kg L-NAME	124 ± 9*						
1 h after 15 mg/kg L-NAME	122 ± 11*						

L-NAME, N-nitro-L-arginine methyl ester; Phe, phenylephrine; SNP, sodium nitroprusside.
* Significantly higher than baseline value (Bonferroni t-test for multiple comparisons, $P < 0.05$).

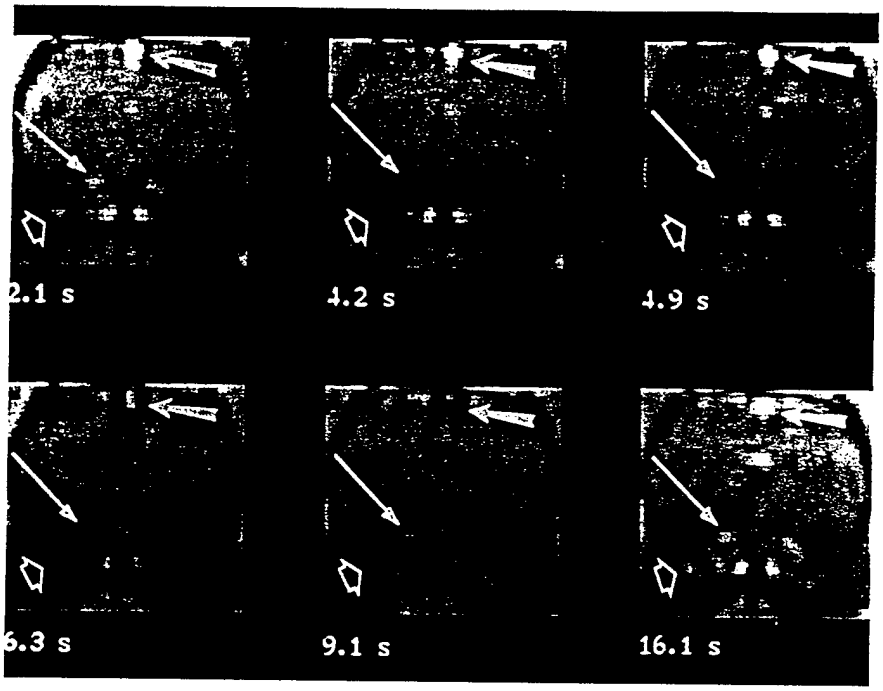

FIG. 1. Dynamic susceptibility contrast images chosen out of 30 gradient-echo images acquired every 0.7 seconds before and after injection of 0.3 mmol/kg gadopentetate dimeglumine. The first image corresponds to 2.1 seconds after injection of the contrast agent, which did not reach the brain yet. The arteries (intracerebral artery: long thin arrow, and artery of the Willis circle: short empty arrow), and the sagittal sinus (thick arrow) are brighter than the cerebral tissue. The arteries become darker 4.2 seconds after injection, followed by the cerebral parenchyma at 4.9 seconds. After 6.3 seconds, the contrast agent reached the sagittal sinus. At 9.1 seconds, the first pass of the contrast agent had left the arteries, which reappeared brighter, while the sagittal sinus was still dark. After 16.1 seconds, the contrast agent had also left the sagittal sinus.

crease after L-NAME injection (95 ± 38% and 86 ± 29% 30 minutes after 3 mg/kg and 15 mg/kg L-NAME, respectively), but did not reach statistical significance because of variance. Sodium nitroprusside infusion restored CBV after the decrease induced by 20 mg/kg L-NAME or increased it to higher values than during the control period (from 81 and 83% to 95 or 137%, respectively in 2 rats). Phenylephrine infusion increased CBV which came back to baseline when the phenylephrine infusion was discontinued (170 and 115% to 105 and 95%, respectively in 2 rats). If the input function correction of $\int C_v(t)dt$ had not been taken into account, the values of $\int C_b(t)dt$ would have shown a trend for increased CBV after 15 mg/kg L-NAME (114 ± 20% after 30 minutes and 108 ± 21% after 60 minutes), contrary to the results found by Equation 4, where CBV is corrected for $\int C_v(t)dt$ (86 ± 29% after 30 minutes and 90 ± 38% after 60 minutes).

MTT_{tissue} was calculated by subtracting MTT_a from MTT_v (Equation 3). There was large interindividual variations when MTT_{tissue} were calculated, but interesting trends were observed. MTT_{tissue} decreased slightly after 3 mg/kg L-NAME (from 1.59 ± 0.61 seconds at baseline to 1.47 ± 0.89 and 1.27 ± 1.02 seconds at 30 and 60 minutes after 3 mg/kg L-NAME respectively) but increased to 2.07 ± 1.06 and 2.00 ± 1.46 seconds at 30 and 60 minutes after 15 mg/kg L-NAME, respectively. Considering that CBF is the ratio of CBV to MTT_{tissue} (Equation 1), the decreased CBV and increased MTT_{tissue} observed after 15 mg/kg L-NAME suggests that CBF was decreased in these conditions. Figure 5 illustrates that the decrease of CBF after 15 mg/kg L-NAME would not have been appropriately recorded if the input function had not been taken into account: CBV/MTT_{tissue} decreased to 66 ± 17% and 72 ± 25% at 30 and 60 minutes after 15 mg/kg L-NAME compared with 100 ± 22% at baseline, while $\int C_b(t)dt/MTT_b$ remained at 93 ± 9% and 91 ± 8% for the same conditions compared with 100 ± 5% at baseline. The smaller numbers of data in L-NAME + SNP and phenylephrine groups did not allow the calculation of mean MTT_{tissue} and CBF, but the individual values of CBF were calculated. They indicated that SNP reversed the decrease of CBF induced by the L-NAME injection, and that phenylephrine infusion increased CBF, which decreased again when the phenylephrine infusion was stopped (Table 2).

DISCUSSION

The present study showed that changes in the physiological state of the rats investigated here can modify the input function. The assumption of a constant input function allows the comparison of different brain regions within one individual at a time. Under constant physi-

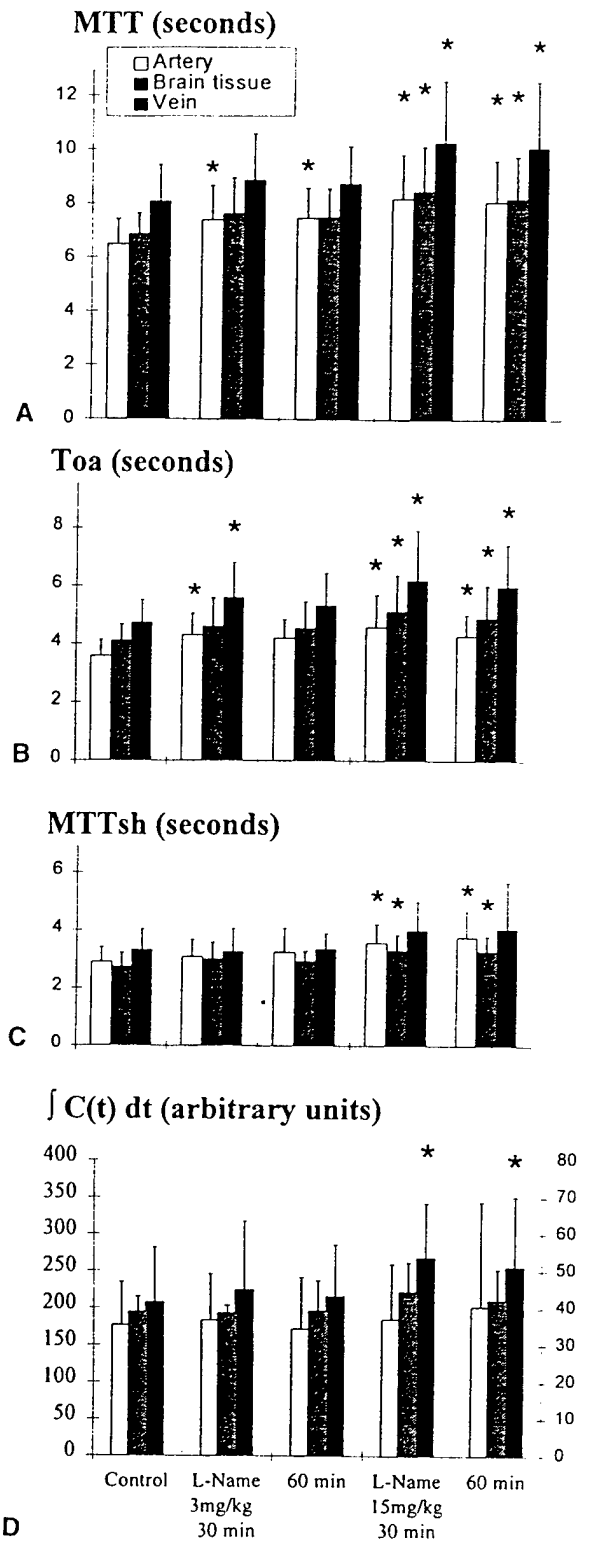

FIG. 2. (A) Mean transit time (MTT), (B) time of arrival (Toa), (C) MTT corrected for Toa (MTTsh), and (D) area of the arterial, brain tissue, and venous concentration-time curves ($\int C_v(t)dt$) in control conditions and after N_ω-Nitro-L-arginine methyl ester (L-NAME) injections. *Parameters significantly increased compared with the control values (Bonferroni t-test for multiple comparisons, $P < 0.05$). In D, the area under the brain curve is indicated on the right side axis.

ological conditions, the validity of this assumption has already been shown by others (Wittlich et al., 1995). This previous investigation also proved that input function can be used as a constant for a group of genetically homogeneous animals, provided the physiological and the imaging conditions of the animals remain unchanged. In an MR human study, input function measured in arteries visible on the scan plane showed a strong interindividual variation, although none of the investigated subjects had a history of intracranial abnormality (Rempp et al., 1994), suggesting that among humans input function differs even if the individuals are subjected to the same conditions.

The modifications of the concentration-time curves observed in the brain tissue and in the sagittal vein after administration of L-NAME were partly caused by their convolution with a modified input function. This was illustrated by the decreased calculated CBV, resulting from an increase of the area under $C_b(t)$, but a stronger increase of the area under $C_v(t)$, used to correct $\int C_b(t)dt$. Ignoring the correction by the input function would have led to the calculation of increased CBV after 15 mg/kg L-NAME.

With this correction, the CBF calculated for 30 minutes after 15 mg/kg L-NAME was reduced to 66 ± 17% of the baseline value. This result is consistent with previous laser Doppler measurements showing that CBF decreased to 67.6 ± 8.1% in rat cerebral cortex after 15 mg/kg L-NAME (Prado et al., 1992) or to 74 ± 2% after 20 mg/kg L-NAME (Smith et al., 1995), with radioactive microsphere measurements showing a global CBF decreased to 74 ± 12% after 20 mg/kg L-NAME (Huang et al., 1994), and with [^{14}C]-iodoantipyrine autoradiography showing a decrease of CBF between 53 ± 3% and 86 ± 6%, depending on the cortical area, after 30 mg/kg L-NAME (Kelly et al., 1994).

The shape and TOA of the input function depends on the way the intravenous contrast agent bolus has been delayed and deformed between the site of injection and the observed feeding artery. The lower dose of 3 mg/kg L-NAME induced a 31% increase of the MABP, close to the 39% increase caused by the higher dose, but less consistent modifications of the concentration-time curves, suggesting that those modifications were more related to the level of nitric oxide synthase inhibition rather than to the consequent hypertension. The reversal of these modifications by SNP infusion would thus be more likely caused by the direct supply of nitric oxide synthase rather than to the reversal of MABP changes. Phenylephrine infusion caused a comparable hypertension, but did not increase TOA, MTT or MTTsh, or even decreased those parameters.

Modifications of cerebral vascular resistance (CVR), and the relative value of CVR compared with the vascular resistance in other organs, could affect the bolus dis-

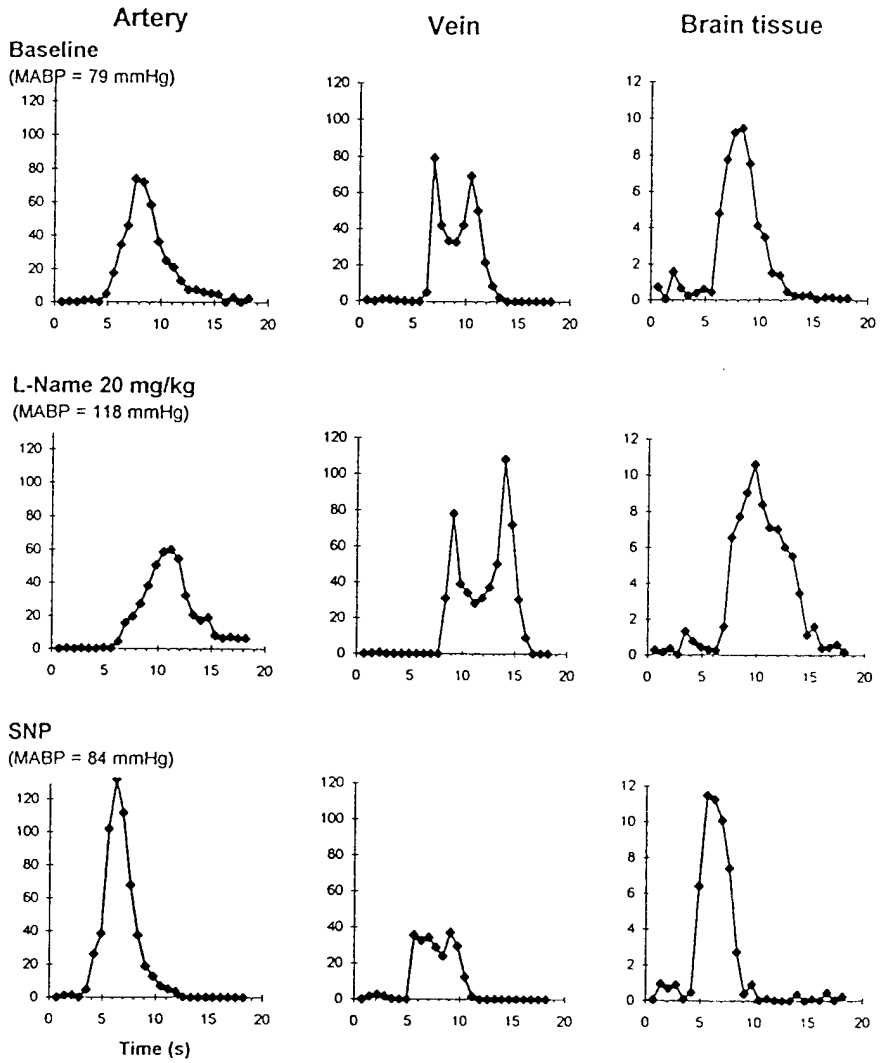

FIG. 3. Arterial, brain tissue, and venous concentration-time curves of a rat administered 20 mg/kg L-NAME followed by sodium nitroprusside (SNP) infusion (0.1 mg/mL, 20 to 40 μL/kg/min). L-NAME increased the MABP, the time of arrival, and mean transit time of all curves, and SNP reversed these modifications. These results indicate that the alterations seen with L-NAME (Fig. 2) were not simply caused by the effect of serial injections.

tribution to brain during its course in the aorta and likely influence the size and shape of the cerebral input function. Calculating CVR as the ratio of MABP to CBF, we found that CVR reached 211% of the control value 30 minutes after 15 mg/kg L-NAME. This increase of CVR induced by L-NAME injection is comparable with the values calculated with MABP and CBF values of previous studies: CVR = 183% (Huang et al., 1994) or 164% (Smith et al., 1995) after 20 mg/kg intravenously, and 156 to 253% depending on the cortical area after 30 mg/kg intravenously (Kelly et al., 1994). However, during phenylephrine infusion, our calculations suggested that CBF actually increased, as would be the case if there was a breakthrough of autoregulation. Consequently, the hypertension induced by phenylephrine infusion was accompanied by a smaller CVR increase than the hypertension induced by L-NAME. The distribution of the contrast agent bolus to the brain would thus be less (or differently) affected, contributing to the difference in input function modifications between both conditions. The trend of increasing CBF with phenylephrine, suggesting a partial breakthrough of autoregulation, would be in agreement with previous laser Doppler measurements of increased CBF in urethane anesthetized rats subjected to phenylephrine (Tsai et al., 1989), and with the previously reported impairment of the cerebral autoregulation during phenylephrine infusion in halothane-anesthetized rabbits (Mutch et al., 1990). The dependence of input function on CVR relative to other vascular beds would also suggest that the measurement of input function in blood samples withdrawn from a peripheral artery, as practiced in single pass CBF measurements techniques, could be misleading if it does not reflect the input function in the carotid bed.

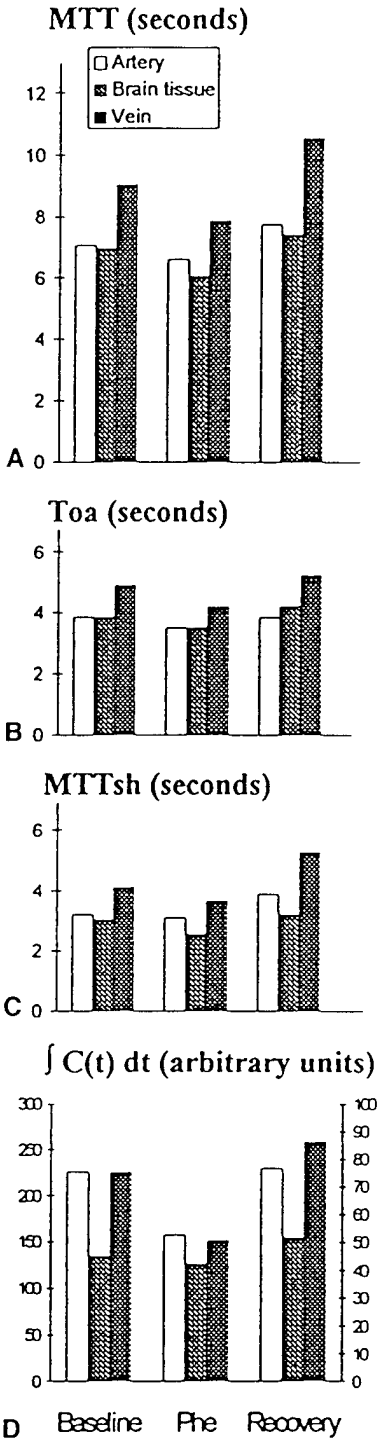

FIG. 4. Mean values of **(A)** mean transit time (MTT), **(B)** time of arrival (Toa), **(C)** MTT corrected for Toa (MTTsh), and **(D)** area of the arterial, brain tissue, and venous concentration-time curves ($\int C_v(t)dt$), during control conditions (baseline), during phenylephrine infusion (Phe), and after stopping phenylephrine (recovery), for the two animals studied. In D, the area under the brain curve is indicated on the right side axis.

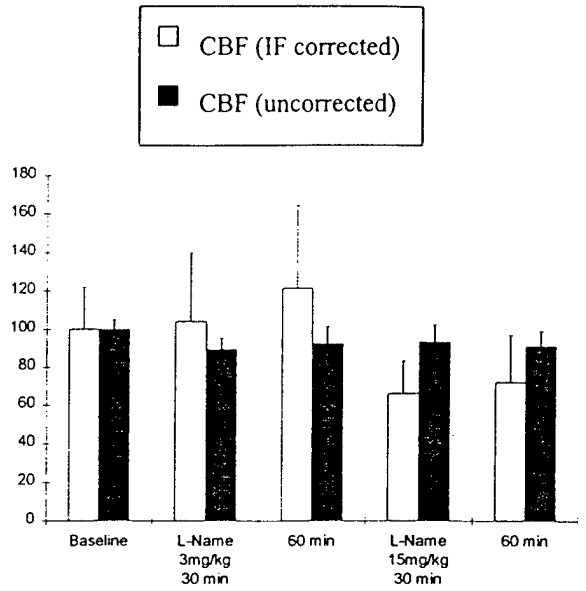

FIG. 5. Assessment of CBF changes after L-NAME injections, with correction by the input function (CBV/MTT_{tissue}) or without this correction ($\int C_v(t)dt$). The displayed results are the ratios of mean values of CBV and MTT_{tissue}, $\int C_v(t)dt$ and MTT_b. Error bars are the SD of these ratios (Raj, 1968).

The indicator dilution theory states that the integrated input function = $\int C_a(t)dt = \int C_v(t)dt$, but the present study found larger areas under the vein curves than under the arterial curves. This finding may be explained by a partial volume effect affecting the arterial pixels more than the venous pixels, because of the smaller size of the arteries compared with the superior sagittal sinus. Although showing larger areas under the curves, $\int C_v(t)dt$ was still apparently underestimated. The ratio of $\int C_b(t)dt$ to $\int C_v(t)dt$ yielded an absolute value of 21 ± 2% for CBV under control conditions, which is too high compared with the values measured previously in rat brains by other methods (3.4% (Shockley, 1988; Kent, 1989)). Thus, volume averaging may not be the only cause of signal loss and both flow and susceptibility effects may be occurring.

Most previous CBF studies calculated CBF by the ratio of CBV to the MTT of the tissue concentration-time curve by first pass bolus tracking. But Hamberg et al. (1993) and Weisskoff et al. (1993) expressed reservations about the use of MTT measured with MRI tissue concentration-time curves for the calculation of CBF by the central volume principle. Weisskoff et al. showed that the first moment of the concentration-time curve of the tracer at the outlet of the system, which relates CBV to CBF, may not be substituted by the first moment of the concentration-time curve measured in the tissue, R(t), which expresses the amount of the bolus that remains in the tissue. They showed that the first moment of R(t) depends on the topology of the vessels. In the present study, we calculated CBF by using MTT_v, the first mo-

TABLE 2. *Estimation of cerebral blood flow**

L-NAME (n = 9)		L-NAME + SNP (n = 2)			Phe (n = 2)		
	Mean CBV / Mean MTT_{tissue} ± SD†		Rat 1	Rat 2		Rat 1	Rat 2
Baseline	100 ± 22	Baseline	100	100	Baseline	100	100
30 min after 3 mg/kg L-NAME	104 ± 36	30 min after 20 mg/kg L-NAME	64	39	Phe	202	269
1 h after 3 mg/kg L-NAME	121 ± 43	L-NAME + SNP	71	74	Discontinuation of Phe	56	105
30 min after 15 mg/kg L-NAME	66 ± 17						
1 h after 15 mg/kg L-NAME	72 ± 25						

L-NAME, N_ω-nitro-L-arginine methyl ester; Phe, phenylephrine; SNP, sodium nitroprusside; CBV, cerebral blood volume; MTT, mean transit time.
* Ratios and SD values were converted in percents of the estimated baseline CBF.
†SD on the ratio of mean CBV to mean MTT_{tissue} were calculated according to Raj (1968). Statistical differences between the estimated CBF were not determined because those values were not the means of individual measurements.

ment of the concentration-time curve in the superior sagittal sinus, i.e. the MTT actually measured at the outlet of the system, in agreement with the central volume principle. But the drawback of using MTT_v (corrected by MTT_a to take into account the deconvolution with input function), is that it gave only the value of a global CBF, and did not allow the calculation of regional CBF in the different anatomical structures of the brain.

Weisskoff et al. (1993) also suggested that the ratio of MTT measured by R(t) could give a reasonable estimate of the relative flow changes either between two regions of the brain with similar vascular physiology, or between the same region before and after some perturbation, if the perturbation changes the vascular structure only moderately. It must be kept in mind also, that in this case, MTT from the input bolus must be subtracted from MTT of R(t). In our study, this estimation could not be calculated because of too small differences between MTT_b and MTT_a. In addition to relative poor temporal resolution, this effect was probably caused by underestimation of MTT_b, because of the low signal to noise ratio of $C_b(t)$ which could have hidden the extremes of the curve into an apparent baseline.

In conclusion, significant changes of the brain input function were found after intravenous injection of L-NAME. These input function changes were not simply caused by an effect on MABP, but possibly involved the complex effects of L-NAME on vascular resistance in the brain and other organs. The important implications of input function modifications for accurate measurements of CBV and CBF were illustrated by calculating their changes after L-NAME injection.

Acknowledgments: We thank Douglas Barstow, Dr. Pitchaiah Mandava, Bernard Kim, Charmaine Rea, and Benoit Leroy for their technical help; and Tatsuo Uchida, MA, Office of Biostatistics, University of Texas Medical Branch, for important statistical advice.

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JOURNAL OF NEUROTRAUMA
Volume 14, Number 4, 1997
Mary Ann Liebert, Inc.

L-Arginine and Superoxide Dismutase Prevent or Reverse Cerebral Hypoperfusion after Fluid-Percussion Traumatic Brain Injury

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ABSTRACT

To determine whether treatment with L-arginine or superoxide dismutase (SOD) would prove effective in reducing cerebral hypoperfusion after traumatic brain injury (TBI), we measured cerebral blood flow (CBF) using laser Doppler flowmetry (LDF) in rats treated before or after moderate (2.2 atm) fluid-percussion (FP) TBI. Rats were anesthetized with isoflurane and prepared for midline FP TBI and then for LDF by thinning the calvaria using an air-cooled drill. Rats were then randomly assigned to receive sham injury, sham injury plus L-arginine (100 mg/kg, 5 min after sham TBI), TBI plus 0.9% NaCl, TBI plus L-arginine (100 mg/kg, 5 min post-TBI), TBI plus SOD (24,000 U/kg pre-TBI + 1600 units/kg/min for 15 min after TBI), or TBI plus SOD and L-arginine. A second group of rats received TBI plus saline, L-, or D-arginine (100 mg/kg, 5 min after-TBI). After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h and CBF was expressed as a percent of the preinjury baseline for 2 h after TBI. Rats treated with saline or D-arginine exhibited significant reductions in CBF that persisted throughout the monitoring period. Rats treated with L-arginine alone or in combination with SOD exhibited no decreases in CBF after TBI. CBF in the SOD-treated group decreased significantly within 15 min after TBI but returned to baseline levels by 45 min after TBI. These studies indicate that L-arginine but not D-arginine administered after TBI prevents posttraumatic hypoperfusion and that pretreatment with SOD will restore CBF after a brief period of hypoperfusion.

Key words: cerebral circulation; free radicals; head trauma; ischemia; L-arginine; laser Doppler; nitric oxide

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) in humans results in reduced cerebral blood flow (CBF) in the first few hours after injury (Bouma et al., 1991, 1992). Although the role played by posttraumatic hypoperfusion in this pathophysiology is not known, evidence of ischemia in most TBI patients (Graham et al., 1978) suggests that reductions in CBF may be important contributors. Early posttraumatic hypoperfusion occurs after experimental TBI in rats (Yuan et al., 1988; Yamakami and McIntosh, 1989, 1991). Moderate central or midline fluid-percussion

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(FP) TBI in rats decreased CBF in the cerebral hemispheres, brain stem, and cerebellum (Yuan et al., 1988). Moderate lateral TBI produced similar decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (Yamakami and McIntosh, 1989, 1991). Most recent measurements of CBF after FP using laser Doppler flowmetry (LDF) confirm earlier microsphere CBF measurements (Muir et al., 1992, 1995).

The causes of significant reductions in CBF after TBI in either patients or experimental animals are not known. Posttraumatic hypoperfusion may result from impairment or destruction of a cerebral vasodilatory mechanism or mechanisms. The endothelium-dependent relaxing factor nitric oxide (NO) is one such cerebral vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). Evidence that inhibition of NO synthesis decreases CBF (Beckman et al., 1991; Tamaka et al., 1991; DeWitt et al., 1992; Pelligrino et al., 1993) suggests a resting cerebral vasodilator "tone" owing to the continuous production of NO. Nitric oxide is inactivated by contact with free radicals such as superoxide (Rubanyi and Vanhoutte, 1986). Superoxide anion radicals are produced by FP TBI (Wei et al., 1981; Fabian et al., 1995), perhaps as a byproduct of trauma-induced increases in prostaglandin synthesis (DeWitt et al., 1988). The superoxide radical may inactivate NO, resulting a reduced CBF after TBI. Free radicals contribute to the pathophysiology of TBI, as the cyclooxygenase inhibitor indomethacin and the free radical scavenger superoxide dismutase (SOD) reduce cerebral vascular dysfunction and endothelial damage after TBI (Wei et al., 1981). Treatment with L-arginine, the substrate for the enzyme NO synthase, increases CBF and reduces infarct volume due to focal ischemia (Morikawa et al., 1992a,b), suggesting that increased production of NO might increase CBF and reduce ischemic damage. In contrast, L-arginine increased infarct size in a suture model of middle cerebral artery occlusion in a rat (n = 3) and L-arginine did not reduce infarct volume or increase CBF after a photothrombotic ischemic insult (Prado et al., 1996). Therefore, the effects of L-arginine on CBF after cerebral ischemia are uncertain and L-arginine effects on CBF after TBI are unknown. To determine whether L-arginine would improve CBF after rodent TBI, we measured CBF using LDF in rats treated with L-arginine or SOD or the combination after FP TBI.

MATERIALS AND METHODS

Animal Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2: room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed in a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline FP TBI was previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma, and a modified Luer-Lok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Rats were then prepared for placement of the laser Doppler flow probe (see below). Isoflurane was lowered to 1.5%, and the rats were connected to the trauma device, and subjected to moderate (2.2 atm) TBI.

Laser Doppler Flowmetry

CBF was measured using LDF. The anesthetized rats were surgically prepared for measurement of relative perfusion as described elsewhere (Haberl et al., 1989). Briefly, the left calvaria lateral and slightly posterior to the injury adapter (see above) was thinned using an air-cooled drill (Dremel, Racine, WI). Using an electrode holder on a stereotaxic headholder (Stoelting Co., Wood Dale, IL), a fiberoptic needle probe (Perimed, Stockholm, Sweden) was placed over the thinned parietal calvaria and carefully positioned away from large vessels visible in the remaining calvaria. The probe emits monochromatic red light (632.8 nm), which is reflected by moving erythrocytes. The power and frequency of the reflected signal, monitored by detectors in the needle probe head, are proportional to the blood volume and blood velocity, respectively. Blood velocity is calculated based upon the Doppler shift created by red blood cells moving in the area perfused by the probe laser and reflected back to the receiver in the same probe. Perfusion is calculated as the product of blood volume and velocity in a 1-mm^3 tissue volume under the probe (Haberl et al., 1989). Measurements were recorded on a PeriFlux PF3 Laser Doppler Perfusion Monitor (Perimed). Values for LDF were compared between rats based on a percentage change from baseline values after the experimental procedure.

Experimental Design

Drug treatment. The dose of L-arginine was chosen because, although doses of 300 mg/kg increased CBF and

reduced infarct volume after middle cerebral artery occlusion (Morikawa et al., 1992a; Tofts and Kermode, 1991), L-arginine also increased CBF and pial arteriolar diameter in control rats. A lower dose was used in the present study (100 mg/kg) to minimize the cerebral vasodilatory effects of L-arginine in uninjured rats. The dose of SOD used in the present studies (24,000 U/kg, i.v. bolus, 5 min pre-TBI, plus 1,600 U/kg min for 10 min starting immediately after TBI) has been reported to improve CBF after TBI in rats (Muir et al., 1995).

Experiment 1. Male Sprague-Dawley rats were prepared as described above and randomly assigned to one of the six groups listed below. A sham group (n = 8) was prepared for FP TBI and LDF, connected to the trauma device, and then removed from the device without injury. A saline-treated group (n = 8) was subjected to moderate TBI (2.2 atm) and then infused with 0.9% NaCl (0.1 ml/min for 10 min). The L-arginine group (n = 8) was subjected to moderate TBI and then L-arginine (10 mg/kg/min for 10 min) administration was started 5 min later. An L-arginine sham group (n = 8) received that same dose of L-arginine but no TBI. The SOD-treated group (n = 8) received a 24,000 U/kg bolus 5 min before moderate TBI, followed by 1600 U/kg/min constant infusion for 15 min after TBI (volume = 33 μL/min). A combination group (n = 8) received both SOD (before and after TBI) and L-arginine (after TBI) in the same doses as the individual SOD and L-arginine groups. After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h; CBF is expressed as a percent of the preinjury baseline 15, 30, 45, 60, 75, 90, 105, and 120 min after TBI. The experimental design for experiment 1 is summarized in Figure 1.

Experiment 2. Male Sprague-Dawley rats were prepared as described above and randomly assigned to one of three groups. A saline-treated group (n = 5) was subjected to moderate TBI (2.2 atm) and then infused with 0.9% NaCl (0.1 ml/min for 10 min). An L-arginine-treated group (n = 5) was subjected to moderate TBI and then L-arginine (10 mg/kg/min for 10 min) administration was started 5 min later. A third group (n = 5) was treated identically to the L-arginine group except for re-

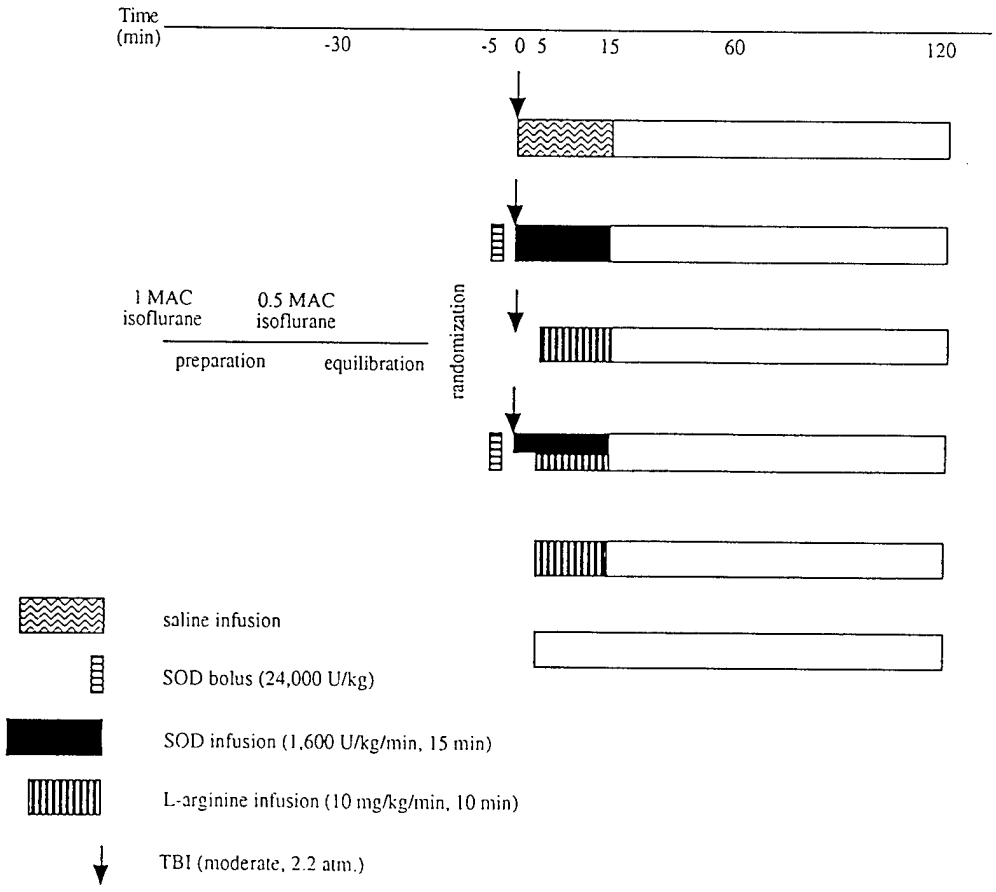

FIG. 1. Schematic of experimental design for experiment 1. The key for the patterned bars is presented in the figure. The open bar represents the monitoring period after TBI and drug infusion. MAC = minimum alveolar anesthetic concentration to produce surgical anesthesia in rats (1.38% isoflurane).

ceiving the inactive stereoisomer, D-arginine (10 mg/kg/min for 10 min), which is not a substrate for NO synthase. After treatment and TBI or sham injury, CBF was measured continuously using LDF for 2 h; CBF is expressed as a percent of the preinjury baseline 15, 30, 60, 90, and 120 min after TBI.

Statistical Evaluations

Data were analyzed using analysis of variance (ANOVA) for a two-factor experiment with repeated measures on time. The two factors were treatment group and time. The number of treatment groups was 6 for experiment 1 and 3 for experiment 2. The time points tested were baseline (before TBI), and 15, 30, 60, 90, and 120 min for CBF and mean arterial pressure (MAP), and before TBI and 60 min after TBI for $PaCO_2$, PaO_2, hemoglobin, and pH. The mean of each time point was compared with the baseline (100% for CBF and the mean for MAP) for each treatment group using Fisher's least significant difference procedure. All values in the text, tables, and figures are expressed as means ± SEM.

RESULTS

Experiment 1

In experiment 1, two rats died during surgical preparation and four rats became progressively hypotensive and died with 15 min of TBI. There were no significant differences in body temperature, $PaCO_2$, PaO_2, or arterial hemoglobin concentrations within any group between pre-TBI baselines and any subsequent measurement interval (Tables 1, 2). In the three groups subjected to moderate TBI, MAP increased within 60 sec of injury and then returned to baseline within 5 min. Except for the acute hypertension period after TBI, there were no significant differences in MAP between the pre-TBI baseline and any measurement interval within any group.

In the sham-injured control rats ($n = 8$) and in the sham-injured rats treated with L-arginine ($n = 8$), CBF remained constant throughout the 2-h measurement interval (Fig. 2). After TBI in the saline-treated group ($n = 8$), CBF decreased significantly ($p < 0.05$) within 15 min after TBI and remained significantly below baseline during the 120-min measurement interval (Figs. 2 and 3). CBF decreased within 15 min after TBI in the SOD-treated group ($n = 8$) but then returned to baseline levels within 60 min after TBI. In the group treated 5 min after TBI with L-arginine ($n = 10$), CBF remained equal to baseline levels throughout the measurement interval. Similarly, CBF did not decrease after TBI in the group treated with the combination of L-arginine plus SOD ($n = 8$).

Experiment 2

In experiment 2, one rat died during surgical preparation and two rats became progressively hypotensive and died within 15 min of TBI. There were no significant differences in body temperature, $PaCO_2$, PaO_2, or arterial hemoglobin concentrations within any group between pre-TBI baselines and any subsequent measurement interval (Table 3). Mean arterial pressure increased within 60 sec of injury and then returned to baseline within 5 min. Except for the brief period of acute hypertension after TBI, there were no significant differences in MAP between the pre-TBI baseline and any measurement interval within any group.

In the saline-treated ($n = 5$) and D-arginine-treated ($n = 5$) groups, CBF decreased significantly ($p < 0.05$) to about 60% of baseline values within 15 min after TBI and remained at these significantly reduced levels for the 120-min monitoring period (Fig. 4). CBF in the group treated with L-arginine ($n = 5$) did not change signifi-

TABLE 1. MEAN ARTERIAL PRESSURE IN SHAM-INJURED RATS TREATED WITH SALINE ($n = 8$) OR L-ARGININE ($n = 8$), OR IN RATS TREATED WITH L-ARGININE ($n = 10$), SOD ($n = 8$) OR L-ARGININE PLUS SOD ($n = 8$) AFTER MODERATE FLUID-PERCUSSION TBI

			Time after TBI					
Variable	*Group*	*Pre-TBI*	*1*	*15*	*30*	*60*	*90*	*120*
MAP[a] (mmHg)	sham	115 ± 5	—	111 ± 5	105 ± 4	106 ± 5	105 ± 4	106 ± 7
	arg-sham	117 ± 6	—	115 ± 7	115 ± 6	115 ± 8	108 ± 8	122 ± 4
	saline	103 ± 7	164 ± 12	110 ± 7	108 ± 8	103 ± 9	97 ± 11	107 ± 11
	SOD	109 ± 7	153 ± 15	116 ± 6	113 ± 5	109 ± 5	109 ± 6	100 ± 8
	L-arginine	115 ± 6	156 ± 13	117 ± 6	117 ± 5	117 ± 5	120 ± 6	106 ± 9
	SODarg	113 ± 2	164 ± 18	118 ± 4	111 ± 4	115 ± 8	113 ± 6	111 ± 8

[a]MAP, mean arterial pressure.

TABLE 2. ARTERIAL BLOOD GASES AND HEMOGLOBIN IN SHAM-INJURED RATS TREATED WITH SALINE (n = 8) OR L-ARGININE (n = 8) OR RATS TREATED WITH L-ARGININE (n = 10), SOD (n = 8), OR L-ARGININE PLUS SOD (n = 8) AFTER MODERATE FLUID-PERCUSSION TBI

Variable	*Group*	*Pre-TBI*	*60 min post-TBI*
$PaCO_2$ (mmHg)	sham	36.6 ± 3.0	33.8 ± 4.7
	arg-sham	31.6 ± 2.0	37.8 ± 3.2
	saline	30.4 ± 4.4	34.3 ± 2.1
	SOD	30.1 ± 4.3	35.8 ± 6.1
	L-arginine	43.2 ± 7.2	41.6 ± 6.9
	SODarg	35.6 ± 3.6	38.2 ± 3.8
PaO_2 (mmHg)	sham	289 ± 9	269 ± 17
	arg-sham	280 ± 11	314 ± 14
	saline	286 ± 13	296 ± 20
	SOD	261 ± 27	287 ± 15
	L-arginine	286 ± 10	278 ± 8
	SODarg	280 ± 19	292 ± 14
pH (mmHg)	sham	7.44 ± 0.03	7.36 ± 0.3*
	arg-sham	7.45 ± 0.02	7.36 ± 0.04*
	saline	7.52 ± 0.01	7.41 ± 0.03*
	SOD	7.44 ± 0.03	7.40 ± 0.04*
	L-arginine	7.44 ± 0.02	7.40 ± 0.01*
	SODarg	7.44 ± 0.02	7.36 ± 0.03*
Hgb (g/dl)	sham	12.5 ± 0.9	12.8 ± 0.6
	arg-sham	13.0 ± 0.6	12.5 ± 0.6
	saline	12.2 ± 0.4	11.7 ± 0.7
	SOD	12.4 ± 0.9	12.4 ± 0.9
	L-arginine	12.8 ± 0.3	11.4 ± 1.4
	SODarg	13.0 ± 0.6	12.7 ± 0.7

*$p < 0.05$ compared with pre-TBI value.

cantly from baseline at any measurement interval after TBI.

DISCUSSION

These studies demonstrated that L-arginine, but not D-arginine, completely prevents posttraumatic hypoperfusion when administered 5 min after moderate FP TBI. L-arginine alone had no effect on CBF. Superoxide dismutase, administered before and after TBI, restored CBF after a 30-min period of hypoperfusion. The combination of L-arginine and SOD prevented posttraumatic hypoperfusion, as did L-arginine alone. Our observations that SOD improves CBF after TBI are consistent with those of Muir et al., who observed that SOD restored CBF nearly to baseline levels within 1 h after moderate FP TBI in rats anesthetized with sodium pentobarbital (Muir et al., 1995).

Posttraumatic hypoperfusion may contribute to the pathophysiology of TBI, as neuropathological evidence of ischemia has been reported in most patients dying after TBI (Graham et al., 1978). In the past, TBI has been reported to result in increases in CBF, termed "luxury perfusion" (Lassen, 1966), or in normal or slightly reduced CBF associated with markedly reduced metabolism (Langfitt and Obrist, 1981). Patients with normal or elevated CBF after severe TBI were significantly more likely to develop intracranial hypertension than patients with CBF levels that corresponded to their reduced cerebral metabolic rates for oxygen (Obrist et al., 1984). Therefore, reduced CBF and posttraumatic ischemia were not thought to play a major role in the pathophysiology of TBI. More recent studies involving CBF measurements within a few hours of injury have shown that nearly one-third of severely injured patients have CBF values below 18 ml/min/100 g and that patients with posttraumatic hypoperfusion have a poorer prognosis than patients with higher CBF levels (Bouma et al., 1992; Miller, 1990). Therefore, evidence that posttraumatic hypoperfusion contributes to secondary injury after TBI suggests that prevention of CBF decreases after injury may be beneficial.

Posttraumatic hypoperfusion is a feature of experimental head injury models. The FP TBI model produces posttraumatic hypoperfusion in cats, pigs, and rats. In cats, CBF does not change significantly in the first hour after injury (DeWitt et al., 1986; McIntosh et al., 1987) but then decreases significantly by 2 h after TBI (McIntosh et al., 1987). In contrast, CBF decreases significantly within 15 min after TBI but returns to baseline within a few hours after TBI in rats and pigs (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1989; Muir et al., 1992; Armstead and Kurth, 1994). Both central and lateral FP TBI produce widespread decreases in CBF in rats. CBF decreases similarly in hemispheres, brain stem, and cerebellum after moderate central FP injury in rats (Yuan et al., 1988). Moderate lateral FP TBI produced significant decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (Yamakami and McIntosh, 1989, 1991). Thus, FP TBI models are suited for studying the mechanisms that contribute to posttraumatic hypoperfusion. In addition, although LDF measures perfusion in a small area of the cerebral hemispheres, CBF values in the cerebral hemispheres seem to reflect the global changes in CBF that occur after FP TBI.

Although the mechanisms contributing to posttraumatic changes in CBF are unknown, one possibility is that TBI interferes with the production of or destroys a cerebral vasodilatory substance or substances. A reasonable candidate for the cerebral vasodilatory agent that is

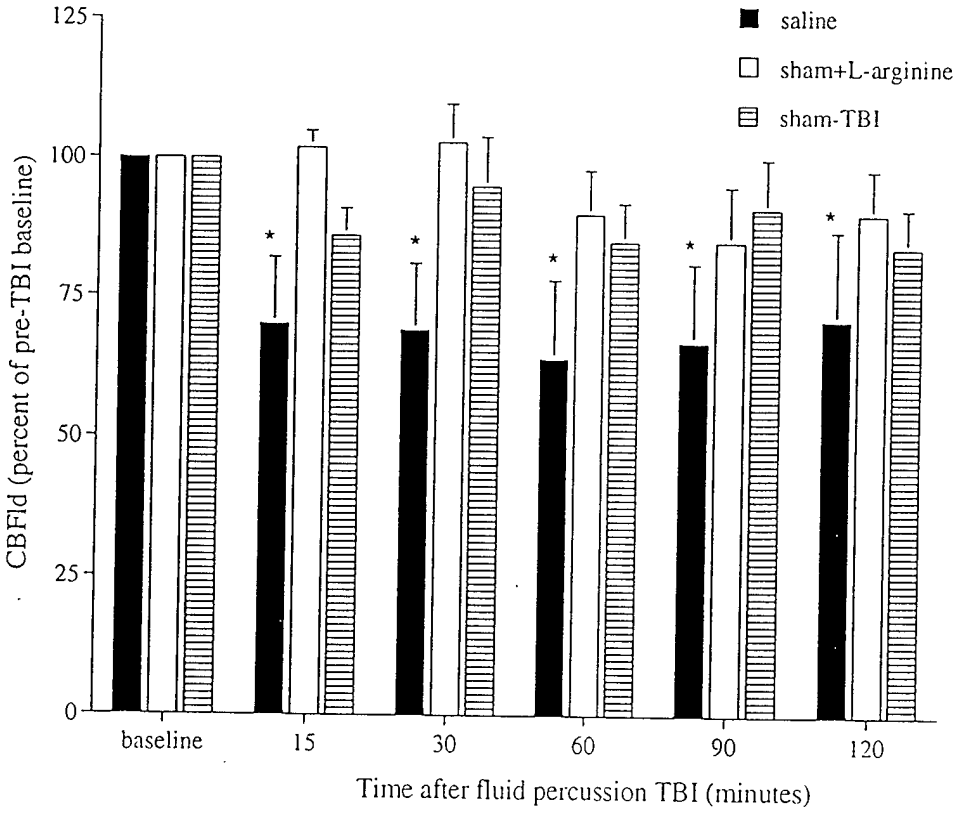

FIG. 2. Laser Doppler cerebral blood flow (CBFld) after traumatic brain injury (TBI) plus saline placebo (saline, $n = 8$) or sham-TBI plus L-arginine (sham + L-arginine, $n = 8$) or sham-TBI with no treatment ($n = 8$). * = significantly ($p < 0.05$) lower than baseline CBF.

affected by TBI is the endothelium-dependent relaxing factor NO. The rodent cerebral circulation exhibits a resting vasodilatory tone mediated by NO (Wang et al., 1992; Pelligrino et al., 1993; Beckman et al., 1991; Tanaka et al., 1991; Faraci, 1991; DeWitt et al., 1992; Kozniewska et al., 1992). Beckman et al. reported that CBF decreased markedly after the infusion of L-nitroarginine hydrochloride (30 mg/kg, i.v.) in halothane-anesthetized rats (Beckman et al., 1991). Tanaka et al., who measured local CBF using iodoantipyrine in awake rats receiving L-nitromonomethyl arginine (L-NMMA, 25 mg/kg, i.v.), found that local CBF was decreased from 20% (substantia nigra) to 33% (hypothalamus) when compared with a saline-treated group (Tanaka et al., 1991). Faraci reported that cerebral arteries and arterioles in barbiturate-anesthetized rats decreased in diameter after topical application of L-NMMA (Faraci, 1991). Kozniewska et al. observed that CBF, measured using intracarotid ^{133}Xenon in rats anesthetized with chloral hydrate, decreased approximately 21% after L-NMMA administration (100 mg/kg, i.v.) (Kozniewska et al., 1992). They also reported that the effects of L-NMMA on CBF could be reversed using L-arginine (300 mg/kg, i.v.) but not D-arginine (300 mg/kg, i.v.). Wang et al., using halothane-anesthetized rats, found that NG-nitro-L-arginine decreased CBF (intracarotid ^{133}Xe) in a dose-dependent manner and that CBF decreases persisted for at least 2 h (Wang et al., 1992). Pelligrino and colleagues reported decreases in regional CBF (radioactive microspheres) of more than 50% after infusion of L-nitroarginine methyl ester (L-NAME, 3 mg/kg/min) in rats anesthetized with 70% N_2O and fentanyl (25 μg/kg/hr) (Pelligrino et al., 1993). Recent autoradiographic studies indicate that L-NAME 30 mg/kg infusion decreases CBF by 17% (parietal cortex) to 49% (hypophysis) across different brain regions in rats (Bonvento et al., 1994). These studies, which were performed using a variety of anesthetic and CBF measurement techniques, consistently demonstrated that the inhibition of NO synthesis decreases CBF and support the hypothesis that there is a resting vasodilatory tone mediated by NO in the rodent cerebral circulation.

Our results, that CBF is preserved after TBI in rats treated with L-arginine but not D-arginine, support the hypothesis that TBI reduces CBF by reducing an NO-mediated cerebral vasodilatory tone. L-arginine has been reported to increase CBF during ischemia (Morikawa et

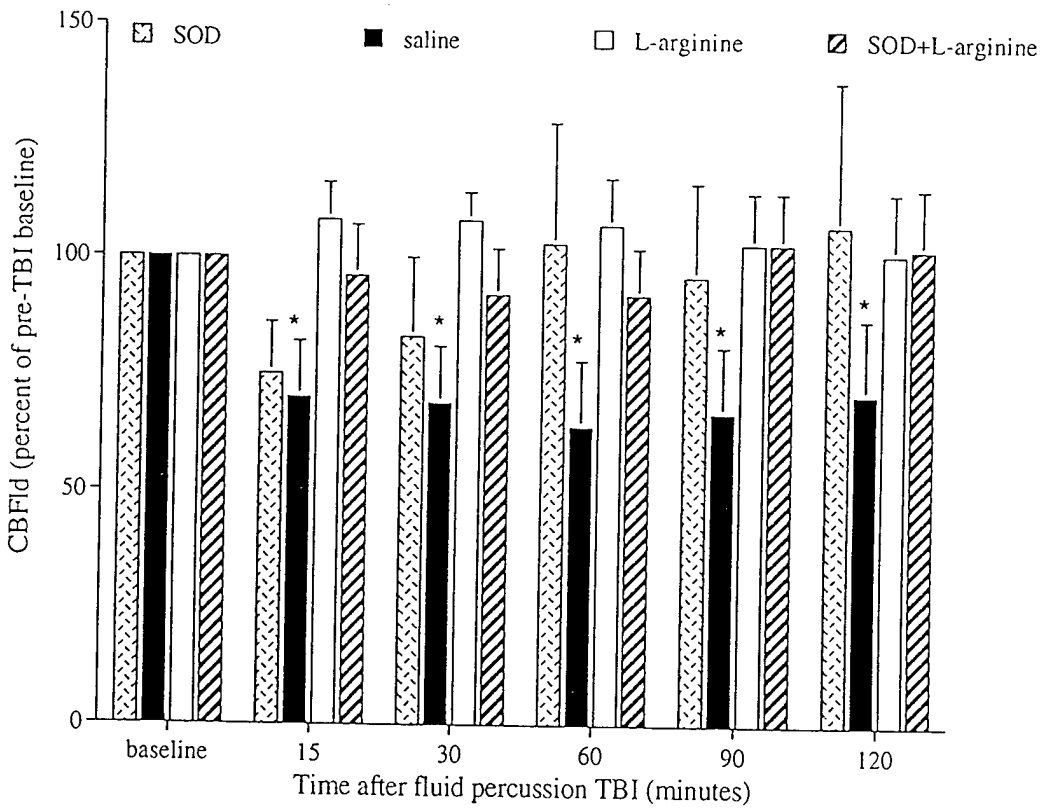

FIG. 3. Laser Doppler cerebral blood flow (CBFld) after traumatic brain injury (TBI) plus superoxide dismutase (SOD) (24,000 U/kg bolus followed by 1,600 U/kg/min infusion for 10 min) (SOD, $n = 8$), TBI plus saline placebo (saline, $n = 8$), TBI plus L-arginine (100 mg/kg) (L-arginine, $n = 10$), or TBI plus SOD and L-arginine (SOD + L-arginine, n = 8). * = significantly ($p < 0.05$) lower than baseline CBF.

al., 1992b; Morikawa et al., 1994) and reduce infarct volume after ischemia (Morikawa et al., 1992b, 1994) in rats. Morikawa et al. also reported that 30 mg/kg and 300 mg/kg L-arginine increased pial arteriolar diameter and that 300 mg/kg L-arginine increased CBF in normal rats (Morikawa et al., 1994). We observed no increase in CBF after the infusion of L-arginine (100 mg/kg) in uninjured rats, perhaps because of the lower dose of L-arginine used in the present study. In addition, CBF responses to L-arginine may be different in rats anesthetized with sodium pentobarbital (Morikawa et al., 1994) than with isoflurane as used in the present study.

If TBI reduces CBF by destroying NO, the mechanism of the effects of TBI on NO remain to be determined. One possibility is that the superoxide anion radical reduces NO levels. Superoxide (O_2^-) anion radical production occurs after FP TBI in cats (Wei et al., 1981; Kontos, 1989; Kontos and Wei, 1992) and rats (Fabian et al., 1995), and reduction of O_2^- activity with SOD or inhibition of O_2^- production with cyclooxygenase inhibitors reduces endothelial damage and restores cerebral vascular reactivity after TBI (Wei et al., 1981; Kontos and Wei, 1992). SOD also restores endothelium-dependent cerebral vasodilatory responses that are markedly attenuated by hemorrhagic hypotension (Szabó et al., 1995). SOD appeared to improve outcome after TBI in Phase II clinical trials (Muizelaar et al., 1993), but more recent results of Phase III clinical trials indicate that SOD may not improve outcome when administered hours after TBI (Young et al., 1996).

NO concentrations, measured using a porphyritic microsensor, decreased during ischemia and then decreased further during reperfusion (Zhang et al., 1995). Superoxide concentrations, measured using a cytochrome C-coated platinum electrode, increase during ischemia and then increase further during reinfusion (Fabian et al., 1995). These observations are consistent with the hypothesis that the superoxide anion radical decreases NO concentrations *in vivo*. Oxygen radical-mediated damage to NO is supported by evidence that antioxidants (mercaptopropionylglycine and catalase) prevent significant decreases in NO_2^- and NO_3^- (stable metabolites of NO) levels that occur during cardiopulmonary bypass in piglets (Morita et al., 1996). In the present study, SOD

TABLE 3. MEAN ARTERIAL PRESSURE (MAP), ARTERIAL BLOOD GASES AND HEMOGLOBIN (Hgb) IN RATS TREATED WITH SALINE (n = 5), L-ARGININE (n = 5) OR D-ARGININE (n = 5) AFTER MODERATE FLUID-PERCUSSION TBI

			Time after TBI (min)					
Variable	*Group*	*Pre-TBI*	*1*	*15*	*30*	*60*	*90*	*120*
MAP	saline	90 ± 12	156 ± 23	91 ± 13	100 ± 15	95 ± 15	98 ± 15	95 ± 15
(mmHg)	L-arg	101 ± 4	121 ± 36	101 ± 3	103 ± 5	101 ± 6	97 ± 8	96 ± 9
	D-arg	108 ± 7	143 ± 25	112 ± 5	116 ± 7	112 ± 3	118 ± 5	116 ± 5
PaO_2	saline	299 ± 5				284 ± 3		
(mmHg)	L-arg	294 ± 6				299 ± 12		
	D-arg	291 ± 16				290 ± 5		
$PaCO_2$	saline	30.5 ± 2.0				38.1 ± 2.9		
(mmHg)	L-arg	38.4 ± 4.9				34.5 ± 1.0		
	D-arg	33.2 ± 2.0				37.3 ± 3.6		
pH	saline	7.48 ± 0.02				7.39 ± 0.02		
	L-arg	7.42 ± 0.04				7.42 ± 0.01		
	D-arg	7.42 ± 0.02				7.40 ± 0.02		
Hgb	saline	12.3 ± 0.6				14.6 ± 0.6		
	L-arg	13.4 ± 0.3				12.9 ± 1.0		
	D-arg	13.1 ± 0.5				12.3 ± 0.4		

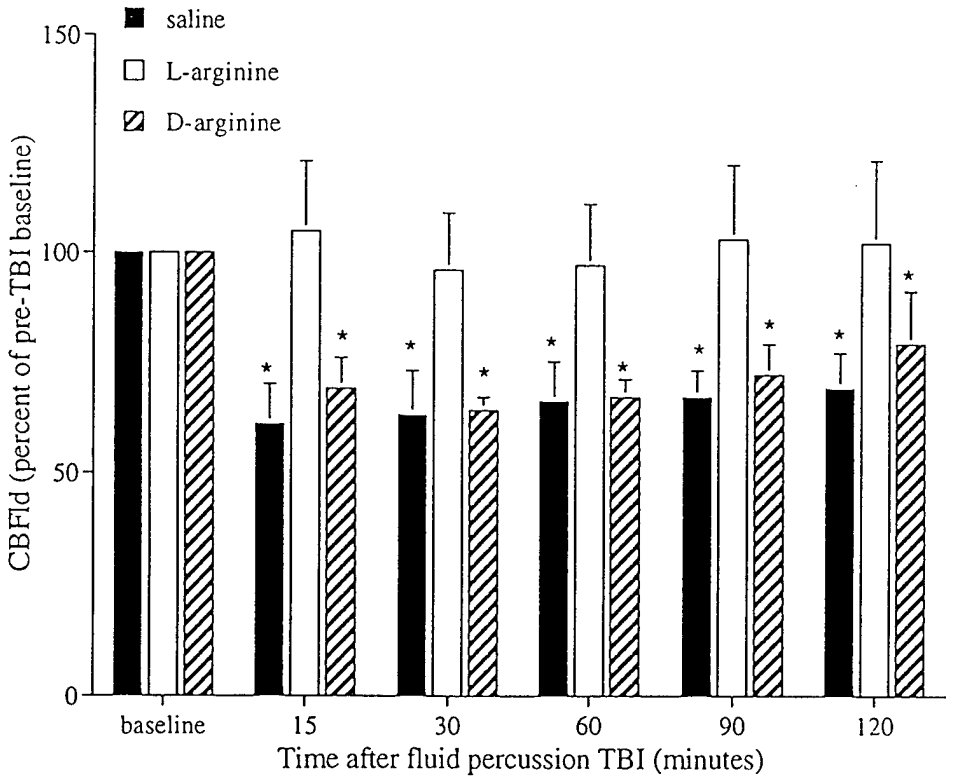

FIG. 4. Laser Doppler cerebral blood flow (CBFld) after TBI plus saline placebo (saline, n = 5), TBI plus L-arginine (100 mg/kg) (L-arginine, n = 5), or TBI plus D-arginine (100 mg/kg) (D-arginine, n = 5). * = significantly ($p < 0.05$) lower than baseline CBF.

did not prevent cerebral hypoperfusion after TBI but restored CBF to baseline after a brief period of hypoperfusion. It is possible that the dose of SOD used in the present studies permitted some transient destruction of NO during the initial burst of O_2^- production that occurs after TBI (Fabian et al., 1995). Superoxide is likely produced during the large increase in prostaglandin production that occurs after TBI in rats (DeWitt et al., 1988). Brain prostaglandin levels increase 20-fold within the first 5 min after moderate FP TBI (DeWitt et al., 1988), perhaps producing levels of O_2^- radicals that were too high to be effectively scavenged by the dose of SOD used in the present studies. The hypothesis that SOD restores CBF by suppressing an initial burst of superoxide production is also consistent with the short plasma half-life of SOD. Native SOD has a plasma half-life of approximately 6 min in rats (Odlind et al., 1988). Therefore, plasma SOD levels would likely have decreased over time after the bolus injection just before TBI. Infusion of SOD immediately after TBI would have slowed the rate of decline in SOD levels, but it is likely that plasma SOD levels decreased markedly after the infusion was stopped. These data suggest that if the initial burst of oxygen radical production can be reduced or if NO substrate levels can be transiently increased, traumatic damage to the cerebral vasculature is minimized and CBF returns to or is maintained at baseline levels.

Superoxide reacts with NO to produce another toxic oxidant, the peroxynitrite anion ($ONOO^-$) (Beckman, 1991), which, when protonated, forms an intermediate species with the reactive properties of nitrogen dioxide and the hydroxyl radical. The relatively high concentrations of SOD and the extremely fast reaction rate of SOD with superoxide normally maintain very low levels of intracellular superoxide anions (Crow and Beckman, 1995). However, the formation of peroxynitrite from superoxide and NO is extremely rapid as well and, if NO is present in sufficient quantities, peroxynitrite formation will predominate (Beckman et al., 1994). What remains to be determined is whether the superoxide radicals produced after TBI are sufficient to overwhelm the available SOD, leading to a shift to peroxynitrite formation (with attendant inactivation of NO). Potentially, excess L-arginine could increase NO concentrations and increase peroxynitrite formation. The concept of conversion of NO to $ONOO^-$ or its destruction by O_2^- through other mechanisms is supported by evidence that SOD markedly increases the stability of NO (Rubanyi and Vanhoutte, 1986). There is clearly a need for further investigations of the role of peroxynitrite in the pathophysiology of TBI.

It is important to note that L-arginine may be acting by a mechanism unrelated to the synthesis of NO. As noted by Morikawa et al., brain intracellular L-arginine concentrations (300–800 μmol/L) are much higher than K_m for NO in the rodent brain (2 μmol/L) (Morikawa et al., 1994; Bredt and Snyder, 1990). Therefore, it is unclear how adding additional substrate to a saturated enzyme system would increase the production of NO enough to overcome decreases in NO that we are suggesting may occur after TBI. It is possible that L-arginine is compartmentalized in certain cells within the neuraxis (Morikawa et al., 1994) and that additional L-arginine would reach cells that have an insufficient supply of substrate. An alternative possibility is that TBI affects NO synthase activity directly and renders L-arginine utilization less effective. Finally, arginine metabolism is complex, and other potentially vasoactive products of arginine metabolism such as polyamines or agmatine may be contributing to the effects of L-arginine on CBF after TBI (Reyes et al., 1994).

These studies demonstrate that posttraumatic hypoperfusion can be prevented by early treatment with the NO precursor, L-arginine, but not by its stereoisomer, D-arginine. Furthermore, they demonstrate that SOD reverses posttraumatic hypoperfusion, suggesting that decreases in CBF that occur immediately after TBI may be due to oxygen radical-mediated destruction of the potent endothelium-derived vasodilatory substance, NO.

ACKNOWLEDGMENTS

The authors thank Christopher Tidwell for technical assistance, Deborah Goodyear for assistance with manuscript preparation, and Kimberly Button for secretarial support. We thank Faith McLellan for editorial review. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. These studies were supported, in part, by NIH grant 19355 to Dr. DeWitt.

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Traumatic brain injury does not alter cerebral artery contractility

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Bukoski, Richard D., Shi Nan Wang, Ka Bian, and Douglas S. DeWitt. Traumatic brain injury does not alter cerebral artery contractility. *Am. J. Physiol.* 272 (*Heart Circ. Physiol.* 41): H1406–H1411, 1997.—Previous studies have shown that traumatic brain injury (TBI) significantly reduces cerebral blood flow determined in vivo and reduces vascular reactivity in the pial circulation measured with cranial window preparations. We have now tested the hypothesis that TBI induces these changes by impairing intrinsic contractile activity of cerebral arteries. Anesthetized rats underwent moderate (2.2 atm) and severe (3.0 atm) midline fluid percussion TBI or sham injury following which posterior cerebral or middle cerebral arteries were isolated and isometric force generation was measured. Moderate (n = 5) and severe (n = 3) trauma had no effect on the magnitude of serotonin- or K^+-induced force generation or sensitivity to serotonin in arteries isolated within 10 min of TBI. Functional disruption of the endothelium of posterior cerebral arteries isolated 10 min after moderate trauma or sham injury caused a reduction in the active tension response to serotonin that was similar in both groups. Blockade of cyclooxygenase with 5 μM indomethacin had no effect on serotonin-induced force generated by vessels with moderate trauma or in sham-treated rats. Acetylcholine induced an endothelium-dependent relaxation of posterior and middle cerebral arteries; the magnitude of the response was unaffected by moderate TBI. To determine whether prolonged in situ exposure of vessels to the traumatized cerebral milieu could reveal an alteration in intrinsic contractility, posterior cerebral arteries were isolated 30 min after TBI; again, no differences in the tension or relaxation responses were observed. It is concluded that midline fluid percussion TBI did not affect contraction or relaxation of proximal middle or posterior cerebral arteries in rats.

brain trauma; vascular reactivity; serotonin; endothelium

TRAUMATIC BRAIN INJURY (TBI) in humans is the leading cause of morbidity and mortality in people under the age of 40 yr in Western society (18). Feline (9, 11, 12, 17, 19, 26, 31, 34), rodent (10, 14, 27, 30, 35–37), and porcine (1) models have provided insight into the mechanisms that contribute to the permanent cognitive and motor deficits that result from TBI. Among the early changes observed after trauma are alterations in cerebrovascular function characterized by cerebral hypoperfusion (3, 35–37) and impaired autoregulatory responses to changes in systemic blood pressure and plasma oxygenation (19–21). TBI also decreases compensatory increases in cerebral blood flow (CBF) that normally occur following isovolumic hemodilution (12, 33). Impairment of these regulatory mechanisms is believed to contribute to deficits in neuronal function or to lead to cell death when arterial hypotension occurs after TBI in humans (8, 23).

The mechanisms by which TBI impairs CBF and cerebral vascular reactivity are not yet fully understood. The majority of studies have been performed using in vivo methods, including the measurement of CBF using microspheres (35–37) and in situ analysis of changes in pial artery diameter using the cranial window preparation (17, 34). Among the more consistent results has been the observation of morphological and functional damage to the endothelium (17, 19). It is therefore logical to propose that our mechanistic understanding of the cellular events that occur in cerebral vascular smooth muscle and endothelial cells after trauma would be facilitated by performing ex vivo analyses of vascular tissue taken from animals after TBI. With this in mind, we initiated the studies that are described in this report. Our primary aim was to test the hypothesis that TBI induces changes in cerebral artery contraction and relaxation that can be detected after the vessel is removed from the animal. The results show that central fluid percussion TBI does not induce intrinsic changes in arterial contractility, and they have important implications regarding the mechanisms of TBI-induced changes in CBF regulation.

METHODS

Animal preparation. Male Sprague-Dawley rats weighing 300–350 g were anesthetized with 1.5–2% isoflurane in 70% air-30% O_2 and surgically prepared for midline fluid percussion injury as previously described (14). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.8-mm hole was trephined into the skull over the sagittal suture, and a modified Luer-lok syringe hub was placed over the exposed dura and bonded in place with adhesive. We then connected the rats to the trauma device and they were subjected to moderate (2.2 atm) or severe (3.0 atm) TBI. Arterial blood pressure, monitored via a polyethylene catheter in one femoral artery, was recorded using an MP100 computer data-acquisition system (Biopac Systems, Goleta, CA).

After injury or sham injury, the rats were exsanguinated via cardiac puncture. The skin over the calvaria was reflected, and the calvaria was removed by cutting through the lateral walls of the orbit, the temporal bone superior to the zygomatic arch, and the parietal and occipital bones. After the calvaria was removed, the brain was chilled by dousing it with ice-cold saline, after which it was excised, with care taken to avoid stretching cerebral vessels at their site of attachment to the cerebral arterial circle (of Willis). Posterior cerebral arteries, including first and second branches, or middle cerebral arteries were removed and cleaned of loosely associated connective tissue.

Isometric force. Isometric force generation of the isolated vessels was determined using established methods (5). Because the vessels were studied at a standardized length, the

diameter of each segment was not systematically measured. However, cerebral arteries from similar animals studied in our laboratory with a cannulated vessel system have an internal diameter of 75–100 μm when pressurized at 100 cmH_2O. Vessels were mounted on a dual-channel wire myograph and maintained in physiological salt solution (PSS) of the following composition (in mM): 130 NaCl, 4.7 KCl, 1.17 $MgSO_4 \cdot 7\ H_2O$, 5 glucose, 1.50 $CaCl_2$, and 15 $NaHCO_3$. When gassed with a mixture of 95% air-5% CO_2, this solution had a pH of 7.4. After an equilibration period of 30 min at 37°C, the axial length was measured using a filar micrometer eyepiece, and the segments were set to their optimal length for force development by construction of an active length-tension curve using solution containing 100 mM K^+ to stimulate active force generation. The composition of this high-K^+ solution was (in mM): 34.7 NaCl, 100 KCl, 1.17 $MgSO_4 \cdot 7\ H_2O$, 5 glucose, 1.50 $CaCl_2$; and 15 $NaHCO_3$. This solution had a pH of 7.4. We denuded some vessels of the endothelium by stroking a human hair in and out of the lumen of the vessel. To verify the status of the endothelium, each vessel was precontracted with a concentration of serotonin (0.3 μM), which induced ~70% of maximal force, and the relaxation response to acetylcholine was assessed. The absence of a relaxation response to acetylcholine was taken to indicate that the endothelium had been functionally disrupted (22). In some vessels the effect of inhibition of cyclooxygenase was assessed by pretreatment of the vessel with 5 μM indomethacin (22). Concentration-response curves were generated by the cumulative addition of the indicated agonist. All responses were recorded in units of millinewtons and normalized to the axial length of the vessel segment and expressed as active tension in millinewtons per millimeter.

Experimental design. One group of experiments assessed the basic reactivity of posterior cerebral arteries isolated from rats that underwent moderate trauma. These vessels were isolated within 10 min of TBI. A second group of experiments assessed the effect of graded levels of trauma and assessed posterior cerebral arteries after either moderate or severe TBI. In a third set of experiments, the effect of moderate trauma on reactivity of middle cerebral arteries isolated within 10 min after trauma was assessed. A fourth group of experiments assessed the effect of a prolonged posttraumatic incubation period after moderate TBI on the reactivity of subsequently isolated posterior cerebral arteries. In this group, vessels were isolated 30 min after the traumatic event.

Data analysis. Results were analyzed for differences using either Student's *t*-test or analysis of variance with a repeated-measures design (SYSTAT software system). A P value of ≤ 0.05 was assumed to indicate a significant difference.

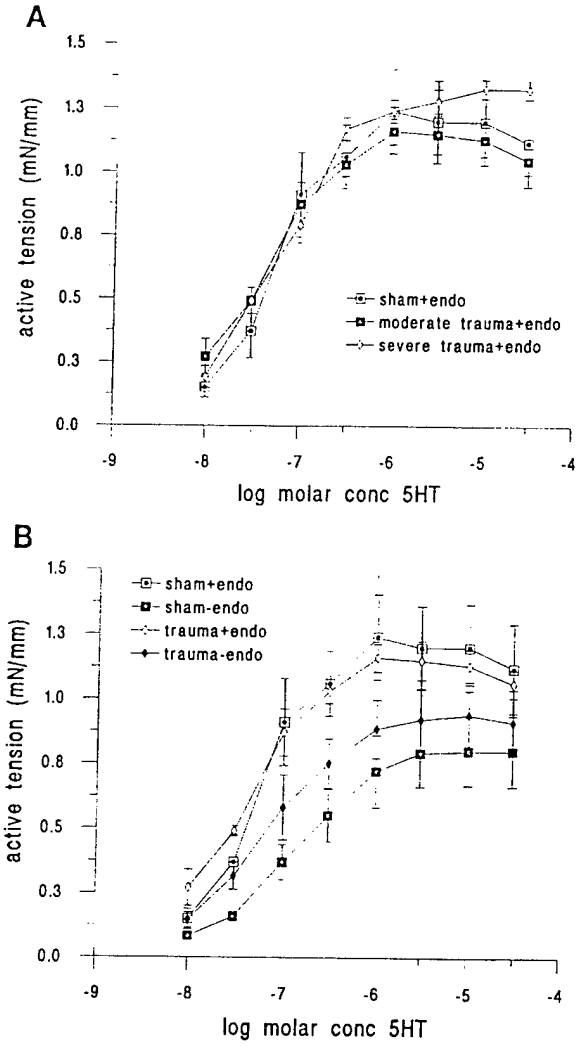

Fig. 1. Effect of trauma on active tension response of posterior cerebral arteries. *A*: effect of moderate or severe trauma on endothelium-intact (+endo) posterior cerebral arteries to cumulative addition of serotonin (5-HT). *B*: effect of endothelial denudation (−endo) on the response to 5-HT of vessels from moderately trauma- and sham-injured animals. Values are means ± SE; $n = 5$ for each group. No significant differences were observed.

RESULTS

In initial experiments, we examined the force responses of posterior cerebral arteries isolated from rats within 10 min of moderate trauma. All vessels were set to their optimal length for force generation. No differences in the active tension (force normalized to axial length of the vessel segment) responses to serotonin (Fig. 1*A*) or to a maximal challenge with 100 mM K^+ (Table 1) were observed. To test the hypothesis that endothelium-independent reactivity was altered, responses were also determined in vessels denuded of endothelium. Endothelial denudation significantly attenuated force generation in vessels from both the trauma- and sham-injured groups, but differences between these groups were not detected (Fig. 1*B*). Vessel

Table 1. *Effect of trauma on tension response to 100 mM K^+*

Vessel	Sham Trauma	Moderate Trauma	Severe Trauma
Posterior cerebral artery (10 min post)	1.77 ± 0.2 (6)	2.00 ± 0.18 (9)	2.19 ± 0.05 (5)
Posterior cerebral artery (30 min post)	2.28 ± 0.37 (5)	2.3 ± 0.11 (3)	
Middle cerebral artery (10 min post)	1.47 ± 0.34 (3)	1.78 ± 0.33 (3)	

Values are means ± SE of active tension response to 100 mM K^+ in mN/mm. Number of separate observations is indicated in parentheses. Post, posttrauma. No differences in the levels of trauma within vessel groups were detected.

Table 2. *Effect of indomethacin on tension response to serotonin before and after moderate trauma*

Vessel/Trauma	Control	Control + Indo	Trauma	Trauma + Indo
Posterior cerebral artery (10 min post)	1.24 ± 0.16 (5)	1.15 ± 0.13 (5)	1.16 ± 0.05 (5)	1.19 ± 0.06 (5)
Middle cerebral artery (10 min post)	0.91 ± 0.24 (3)	0.87 ± 0.22 (3)	1.15 ± 0.13 (3)	1.3 ± 0.22 (3)
Post cerebral artery (30 min post)	1.8 ± 0.11 (4)	1.72 ± 0.22 (4)	1.61 ± 0.14 (6)	1.66 ± 0.13 (6)

Values are means ± SE of the active tension response to K^+ in mN/mm. Number of separate observations is indicated in parentheses. Indo, indomethacin; post, post moderate trauma. No effect of indomethacin was detected in any of the groups.

segments were also studied after inhibition of cyclooxygenase by pretreatment of the segments with 5 μM indomethacin to test the hypothesis that vasoactive prostanoid production was altered. Pretreatment with indomethacin had no effect on the active tension response of either the sham-injured or moderately trauma-injured animals (Table 2).

The relaxation response of these posterior cerebral arteries to acetylcholine after precontraction with serotonin was assessed. As shown in Fig. 2, acetylcholine caused a dose-dependent relaxation of precontracted posterior cerebral arteries that was not different between the sham- and trauma-injured animals and was ablated by removal of the endothelium.

Because these results indicated that moderate TBI was without effect on agonist-induced force generation or relaxation, we considered the possibility that a greater level of trauma would be able to induce a persistent change in vascular reactivity. Posterior cerebral arteries were isolated from animals within 10 min of their undergoing severe (3.0 atm) brain trauma. As with the moderately trauma-injured group, the responses of vessels to serotonin in the severely trauma-injured group (Fig. 1*A*) or to K^+ (Table 1) were not different from those in the sham-injured group.

Because posterior cerebral arteries did not show significant alterations in reactivity after moderate or severe TBI, we considered the possibility that regional differences in reactivity may be induced by TBI and assessed the effect of moderate trauma on the reactivity of middle cerebral arteries. Vessels were again isolated within 10 min of the moderate injury and prepared for measurement of isometric force generation. No differences in either the contractile responses of the vessels to serotonin (Fig. 3*A*) or K^+ (Table 1) or in the relaxation responses to acetylcholine (Fig. 3*B*) were detected (n = 3 per group).

As a final consideration, we tested the hypothesis that prolonged in situ exposure of vessels to substances liberated from parenchymal elements or circulating factors induced by trauma might significantly alter the

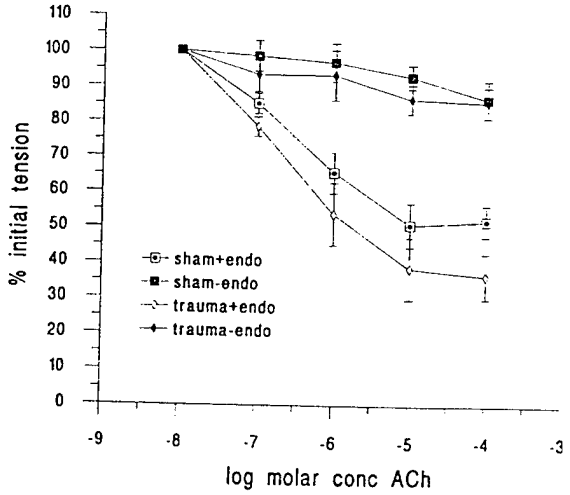

Fig. 2. Effect of moderate trauma on relaxation response of 5-HT-contracted posterior cerebral arteries to cumulative addition of acetylcholine (ACh). Values are means ± SE; n = 5 for each group. No significant differences were observed.

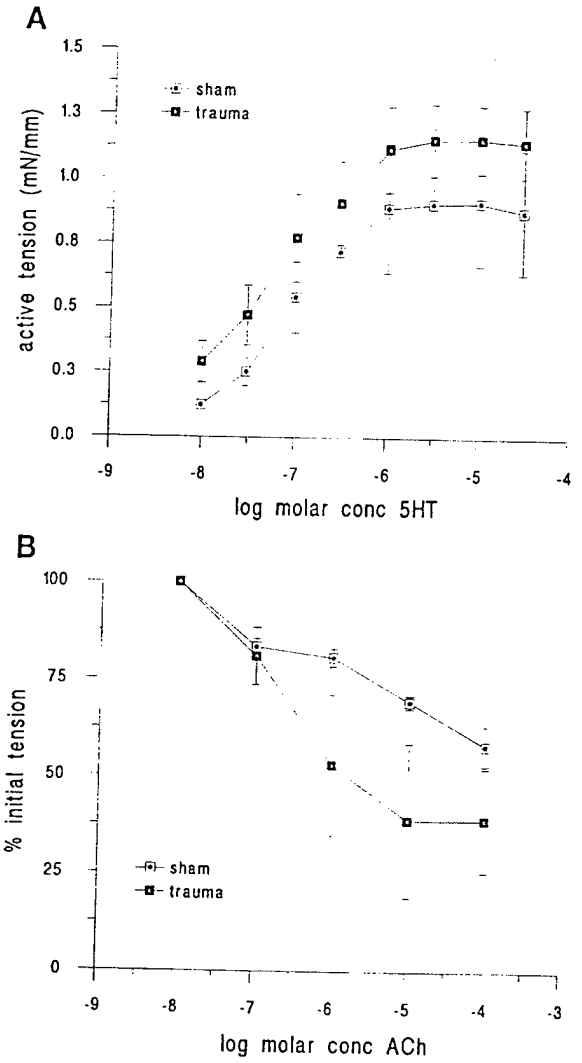

Fig. 3. Effect of moderate trauma on middle cerebral arteries. *A*: effect of moderate trauma on response of endothelium-intact middle cerebral arteries to cumulative addition of 5-HT. *B*: effect of moderate trauma on relaxation response of 5-HT-contracted middle cerebral arteries to cumulative addition of ACh. Values are means ± SE; n = 3 for each group. No significant differences were detected.

intrinsic contractility of cerebral arteries. In these experiments, moderate brain trauma was performed, and the animal was maintained in the experimental apparatus for a 30-min period, following which posterior cerebral arteries were isolated and prepared for analysis of isometric force generation. As with the previous experiments, moderate trauma with prolonged incubation had no effect on the contractile responses of vessels to serotonin (Fig. 4*A*) or K^+ (Table 1) or on the relaxation response to acetylcholine (Fig. 4*B*).

DISCUSSION

On the basis of previous reports that both vasoconstrictor (1, 13, 15, 19, 28, 34) and vasodilator (9, 11, 17, 19–21, 34) responses of the cerebral circulation are impaired by TBI, we tested the hypothesis that TBI induces changes in cerebral artery reactivity that can be detected ex vivo. The major new finding of the present study is that midline fluid percussion TBI does not impair the intrinsic contractile ability of subsequently isolated cerebral arteries. In our initial experiments, the effect of moderate trauma on reactivity of isolated posterior cerebral artery segments was assessed. TBI had no effect on the force response to either serotonin or K^+ (Fig. 1). Moreover, TBI had no effect on the endothelium-dependent dilator response to acetylcholine, although removal of the endothelium significantly impaired the magnitude of force generation (Fig. 2). Therefore, our results indicate that neither endothelium-dependent nor endothelium-independent responses were affected by central fluid percussion injury (FPI).

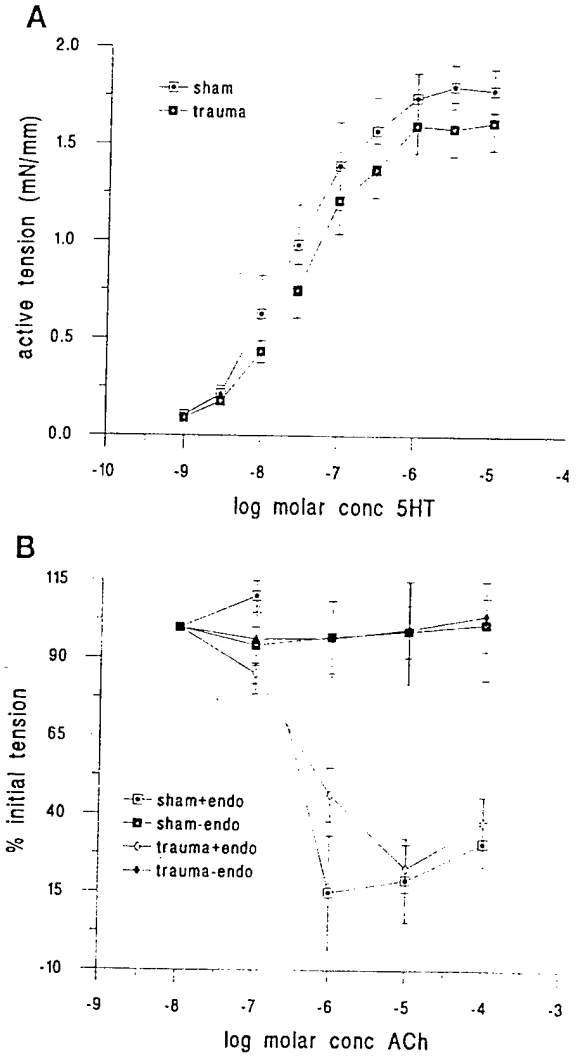

Fig. 4. Effect of posttraumatic in situ incubation on vessel reactivity to 5-HT (*A*) and ACh (*B*). Vessels were isolated 30 min after moderate brain injury and studied with intact endothelia. Values are means ± SE; $n = 3$ for each group. No significant differences were observed.

Regional differences in the degree of impairment of vasodilatory responses to hemorrhagic hypotension can occur after moderate TBI. For example, anterior brain regions in cats exhibit more severe impairment of autoregulation than regions such as the occipital lobes and brain stem (11). We therefore tested the hypothesis that responses in the middle cerebral artery, which supplies more anterior brain regions, may be affected. Our results showed, however, that neither vasoconstrictor responses to serotonin nor the vasodilator responses to acetylcholine are altered by moderate TBI (Fig. 3). Thus regional differences in the arterial responses after moderate, central FPI were not detected in the ex vivo analysis.

FPI has been studied using midline (14), parasagittal (13), and lateral (27) placement of the craniotomy for attachment of the head-injury device. Schmidt and Grady (32) compared blood-brain barrier damage (horseradish peroxidase extravasation) in the three models but did not examine vascular structural damage or cerebral vascular reactivity. Dietrich et al. (13) reported endothelial discontinuities and constricted arterioles in subcortical white matter, hippocampus, and lateral thalamus, but comparable detailed light and electron-microscopic histological analyses have not been performed after midline or lateral FPI. Despite the absence of histological assessments of the effects of midline FPI, other evidence suggests that traumatic effects on CBF and cerebral vascular reactivity are similar after both midline and lateral FPI. CBF decreases similarly in the hemispheres, brain stem, and cerebellum after moderate central FPI in rats (37). Moderate lateral FPI produced significant decreases in CBF in the brain stem, cerebellum, diencephalon, and frontal and parietal cortices on both (i.e., injured and uninjured) sides of the rodent brain (35–37). Although regional autoregulation has not been assessed in rats after TBI, moderate central FPI impairs autoregulation in all 46 brain regions studied in cats (10). Therefore, CBF studies suggest that the effects of moderate, central FPI on the cerebral circulation are not confined to the brain stem. However, we cannot exclude the possibility that TBI affected vessels of a different size or location from those studied or the possibility that lateral FPI may impair the agonist-induced vasoconstrictory or vasodilatory responses that were not affected by central FPI.

It is also recognized that cerebral vascular responses are impaired to a greater degree as the level of TBI increases. Vasoconstrictor responses to hypocapnia in the feline pial arteries and arterioles are reduced by moderate (2.7 atm) TBI and were completely abolished by severe TBI (3.4 atm) (35). Moreover, autoregulatory vasodilatory responses to progressive hemorrhagic hypotension in feline pial arteries and arterioles are reduced by mild (1.6 atm) TBI, whereas higher levels of TBI result in cerebral arterial vasoconstriction during hypotension (35). In the present study, severe TBI (3.0 atm) did not reduce vasoconstriction to serotonin or vasodilatory responses to acetylcholine (Fig. 3). Therefore, the level of TBI has no effect on intrinsic contractile properties of cerebral arteries studied ex vivo.

With continuous monitoring of posttraumatic CBF using laser Doppler flowmetry, Muir et al. (30) reported that CBF decreases gradually during the first 20–30 min after TBI in rats. These observations suggest that traumatic injury to the cerebral vasculature may develop over time. To determine whether there is a gradual impairment of cerebral vascular contractile or dilatory responses after TBI, posterior cerebral arteries were isolated 30 min post-TBI, when CBF decreases are the most pronounced (30, 35). No alterations in contractility or relaxing ability were noted (Fig. 4), indicating that if an injury process develops slowly after TBI in vivo it does not affect fundamental responses to applied agonists in pial arteries.

Although these findings do not support the hypothesis that changes in agonist-mediated vascular reactivity contribute to trauma-induced impairment of cerebral blood flow, one caveat needs to be considered: in addition to agonist-induced contraction and relaxation, cerebral arteries have a significant component of myogenic reactivity (6, 29). It is therefore possible that changes in myogenic reactivity are induced by TBI that would not be detected by the protocols we used. This possibility remains to be examined.

Our results suggesting that the intrinsic mechanisms that regulate vascular reactivity of small cerebral arteries are not altered by TBI have several important implications. One is that alterations in reactivity observed in vivo after brain trauma may be the result of the proximity of the cerebral arteries to the underlying parenchyma of the brain. Factors that need be considered include perivascular innervation, which could modulate reactivity, and exposure to vasoactive factors elicited by trauma from brain parenchyma or arriving at the cerebral circulation via the systemic circulation. Perivascular sympathetic fibers contribute to maintenance of autoregulatory vasoconstriction in response to acute hypertension (2, 7) and, in addition to sympathetic fibers, there are myriad types of perivascular nerve fibers whose function are only partly understood (4). Vasoactive factors produced by TBI include prostanoids (12) and other arachidonic acid metabolites (16), oxygen free radicals (24), endogenous opioids (24, 26), and a variety of other agents (25). Our results also suggest that cerebral arteries remain fundamentally intact and reactive and that proper cerebral vascular function could be restored given proper therapeutic intervention to restore the milieu in which the cerebral circulation exists.

The authors acknowledge the expert technical assistance of Kim Miller and the editorial assistance of Faith McLellan and Wilma Frye.

We thank our colleagues in the Departments of Medicine and Anesthesiology for support of the Hypertension and Vascular Research Laboratories and the Charles R. Allen Research Laboratories, respectively.

This work was funded, in part, by a grant from the National Institute of Neurological Disorders and Stroke (NS-19355).

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Received 18 June 1996; accepted in final form 17 September 1996.

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JOURNAL OF NEUROTRAUMA
Volume 15, Number 6, 1998
Mary Ann Liebert, Inc.

The 21-Aminosteroid U-74389G Reduces Cerebral Superoxide Anion Concentration Following Fluid Percussion Injury of the Brain

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ABSTRACT

We examined the effects of the 21-aminosteroid antioxidant U-74389G (16-desmethyl-tirilazad) on the concentration of extracellular superoxide anion following fluid percussion traumatic brain injury (TBI) measured by a cytochrome *c*–coated electrode and on local cerebral perfusion (CBF_{ld}) measured by laser Doppler flowmetry (LDF). U-74389G in a dose of 3 mg/kg reduced superoxide anion concentrations 60 min after TBI significantly but had no significant effect on CBF_{ld}. These results indicate that reduction of CBF after TBI can be dissociated from superoxide anion production. Persistent ischemia may limit neuroprotection efficacy and may contribute to divergent outcome results in clinical and animal trials using agents to modify reactive oxygen species.

Key words: 21-aminosteroids; cerebral blood flow; oxygen radicals; rat; traumatic brain injury

INTRODUCTION

THE CENTRAL NERVOUS SYSTEM is especially vulnerable to injury from reactive oxygen species (ROS) because of the relative lack of antioxidant defenses, the abundance of stored iron (Zaleska and Floyd, 1985), and polyunsaturated lipids (Watson and Ginsberg, 1988). Tissue injury mediated by free radicals is thought to contribute significantly to tissue injury due to cerebral ischemia, cerebral trauma, and other injuries (Nelson et al., 1992).

Cerebral injury apparently initiates a series of events that produces delayed neuronal death, and coniderable attention has been focused on determining the nature of these posttraumatic events and formulating a means of ameliorating the damage that these events produce. The production of oxygen free radicals in tissue is thought to be one possible mediator of posttraumatic brain injury (Wei et al., 1981). Oxygen radical scavengers have been demonstrated to ameliorate the effects of fluid percussion traumatic brain injury (TBI), such as vascular abnormalities (Kontos and Wei, 1986; Ellis et al., 1991), hyoperfusion (Muir et al., 1995; Bukoski et al., 1997), disruption of the blood-brain barrier, and lipid peroxidation (Hall et al., 1993; Smith et al., 1994). Fluid percussion TBI results in a 20-fold increase in brain prostaglandin levels (DeWitt, et al., 1988; Ellison et al., 1989; Ellis et al., 1991). These marked increases in eicosanoid production are likely associated with increases in oxygen radicals as a byproduct of arachidonic acid metabolism. Production of free radicals has been detected in the brain following TBI using nitroblue tetrazolium (Kontos and Wei, 1986; Povlishock and Kontos, 1992), and with salicylate trapping (Smith et al., 1994). Evidence of free radical production and lipid peroxidation, which results from the reaction between lipid dienes and the hydroxyl radi-

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cal, has been observed within minutes of a traumatic insult (Braughler and Hall, 1989; Hall et al., 1993; Smith et al., 1994; Sen et al., 1994).

A series of 21-aminosteroids has been developed and tested for its ability to inhibit peroxidation of lipids in tissue following various types of injuries (Hall et al., 1991). The 21-aminosteroids include tirilazad and U-74389G (16-desmethyl-tirilazad). This series of compounds appears to have free radical scavenging and membrane stabilizing activities, but the precise nature of their neuroprotective activity is not known (Hall et al., 1991). The effect of pharmacological agents on free radical production and the importance of free radical scavenging activity in neuroprotection has been difficult to determine in part because of the difficulties involved in measuring free radicals *in vivo*.

McNeil et al. (1989, 1992) reported using cytochrome *c*–coated electrodes for the electrochemical measurement of superoxide *in vitro*, and we have subsequently adapted this technique for *in vivo* detection of the superoxide anion (Fabian et al., 1995). In this report, we describe the use of cytochrome *c*-coated electrodes for reagentless, *in vivo* measurement of superoxide production after moderate fluid percussion TBI in the rat. We compare these measurements in the same animals with cerebral blood flow (CBF) measured by laser Doppler flowmetry in animals treated with U-74389G.

MATERIALS AND METHODS

Fabrication and Calibration of the Cytochrome c Electrode (CC-PACE)

The fabrication of the cytochrome *c*–coated electrode has been described elsewhere (Fabian et al., 1995), using a modification of the method of McNeil et al. (1992). Electrodes were constructed of cytochrome *c* adsorbed onto platinized carbon electrode (PACE) material (EFCG S type material, E-Tek, Natick, MA). Uniformity of the electrodes was assured by testing their responsiveness to superoxide anion generated by the xanthine oxidase—xanthine system, as describe previously (Fabian et al., 1995). Electrodes removed from animals were also tested in this way after experiments to determine loss of sensitivity. We have previously shown that these electrodes do not respond to the hydroxyl radical or nitric oxide *in vitro* and that the signal can be inhibited by local or systemic injection of superoxide dismutase (SOD) (Fabian et al., 1995).

Superoxide and Cerebral Perfusion Measurement with Trauma

All experimental protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch. Sprague-Dawley rats of either sex (approximately 450 g) were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2 and room air (50:50) using a volume ventilator (EDCO Scientific Inc., Chapel Hill, NC). Polyethylene cannulas were placed in both femoral arteries and in one vein for arterial blood pressure recording and for drug infusion. Rectal temperature was monitored using a tele-thermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). A 4-mm craniotomy was trephined over the sagittal suture midway between the lambda and the bregma. A plastic adapter for the fluid percussion injury device was cemented into the craniotomy with cyanoacrylic. The pial surface on the left was exposed through a craniotomy positioned 2 mm lateral to the sagittal suture and 2 mm anterior to the bregma. The PACE electrode was juxtaposed to the pial surface, positioned so as to occlude the craniotomy, and held in place with cyanoacrylate. A platinum wire counter-electrode was positioned contralaterally in the subarachnoid space, and a silver chloride reference electrode was inserted into the subarachnoid space through a small craniotomy 3 mm anterior to the working electrode.

The calvarium was thinned posterior to the trauma device adapter to allow for placement of the laser Doppler probe for measurement of CBF as described elsewhere (Haberl et al., 1989). Briefly, the left calvaria lateral and slightly posterior to the injury adapter, 1.5 mm posterior to the PACE electrode, were thinned with an air-cooled drill (Dremel, Racine, WI). By use of an electrode holder on stereotaxic headholder (David Kopf), a fiberoptic needle probe (Perimed, Stockholm, Sweden) was placed over the shaved parietal calvaria and carefully positioned away from the large vessels visible in the remaining calvaria. Perfusion was calculated as the product of blood volume and velocity in a 1 mm:tissue volume under the probe. Measurements were recorded with a PeriFlux PF3 Laser Doppler Perfusion Monitor (Perimed, Stockholm, Sweden) and were compared between rats based on a percentage change from baseline values after experimental procedures (Haberl et al., 1989).

In some animals ($n = 3$) intravenous injections of superoxide dismutase (SOD, Sigma Chem. Co., St. Louis, MO), 25,000 U/kg in 0.5 ml saline, were made following fluid percussion TBI as a control of specificity of electrode current changes for superoxide to confirm that the electrode current signal was inhibited.

The electrodes and the trauma device adapter were then covered with dental acrylic. After surgical preparation, the isoflurane was lowered to 1.4% in O_2 and air, 50:50; and arterial pH, $PaCO_2$, and PaO_2 were monitored and

maintained within normal limits using ventilatory rate and volume adjustments. The supplemental O_2 in the gas mixture was used to reduce the possibility of hypoxia if pulmonary edema occurred after TBI. Relative CBF_{ld} was monitored continuously. After the dental acrylic had hardened, the rats were connected to the fluid percussion injury device described in detail elsewhere (Dixon et al., 1987). The rats were subjected to moderate fluid percussion injury (2.2 atm), and electrode current, blood pressure, and cerebral perfusion were monitored continuously for at least 90 min.

Treatment with U-74389G

Groups of animals subjected to head trauma were treated with either 3 mg/kg U-74389G as 2 mg/ml of carrier (20 mM sodium citrate, 77 mM NaCl, pH 3.0, $n = 6$) or with carrier only (2.5 ml/kg, $n = 6$). Drug and carrier, or carrier only, was injected intravenously as a bolus over 3–5 min, 15 min prior to fluid percussion injury.

Statistics

Means were compared using an unpaired two-tailed t test with a correction for multiple comparisons. All values in the text, table, and figures are expressed as mean ± standard error of the mean (SEM).

RESULTS

There were no significant differences in PaO_2, $PaCO_2$, pH, or arteriolar hemoglobin levels between treatment groups or between pre- and posttrauma measurements (see Table 1). Recordings of mean arterial blood pressure (MAP) and CBF_{ld} for drug-treated and carrier-treated groups are summarized in Figs. 1 and 2, respectively. All values were normalized to pretrauma baseline levels. There was a trend toward an increase in blood pressure following TBI in the drug-treated group that did not reach statistical significance.

CBF_{ld} decreased significantly in both groups and remained depressed for about 1 h before gradually rising, but there was no significant difference between the groups. Changes in CC-PACE and BSA-coated electrode currents are summarized in Fig. 3. There was an increase in current in the CC-PACE electrode, peaking at about 60 min after trauma. Superoxide anion production in the U-74389G group was significantly less than in the carrier-treated group ($p < 0.001$). This difference tended to increase with time. In some animals, a slight shift in the current baseline took take place with the application of trauma, and measurements were made relative to the corrected baseline.

Recordings made using BSA-coated electrodes showed a brief (5- to 10-min) shift in current following TBI, usually a reduction in the oxidizing current followed by an increase, but currents returned to baseline levels after that. As a further control for specificity of the electrode signal to the superoxide anion radical, intravenous injections of SOD, 25,000 U/kg, were administered 30 min after fluid percussion TBI to a subset of the animals, resulting in a maximum reduction of the oxidizing current change of 71 ± 3.3% ($n = 3$). The effect of injection of SOD on the oxidizing current was brief, lasting about 10 min.

DISCUSSION

The cytochrome *c* electrode, which was capable of measuring superoxide levels on a minute-by-minute basis, demonstrated that TBI resulted in increases in superoxide radical production. This increase in the superoxide signal was attenuated by pretreatment with U-74389G.

The lazaroids are known to act as free radical scavengers, and they may react with superoxide and other radicals to decrease their overall concentration *in vivo*. They also serve to stabilize cellular membranes and may by this mechanism interrupt many of the events leading to

TABLE 1. ARTERIAL BLOOD GAS AND HEMOGLOBIN MEASUREMENTS

Treatment group	*pH*	*PaO_2 (mm Hg)*	*$PaCO_2$ (mm Hg)*	*Hgb (g/dl)*
Drug				
Pre-TBI	7.45 ± 0.03	281.5 ± 5.3	38.1 ± 2.8	12.4 ± 1.2
Post-TBI	7.34 ± 0.02	287.4 ± 7.6	44.3 ± 2.5	13.9 ± 0.8
Carrier				
Pre-TBI	7.38 ± 0.41	276.6 ± 7.0	43.8 ± 1.3	13.7 ± 0.2
Post-TBI	7.36 ± 0.01	283.3 ± 2.8	43.1 ± 2.5	13.9 ± 0.9

Values plus or minus SEM. TBI, traumatic brain injury. Drug and carrier only treated groups (see text). Pre-TBI samples were taken 15–30 min prior to fluid percussion TBI, and post-TBI samples were taken 20–60 min after fluid percussion TBI.

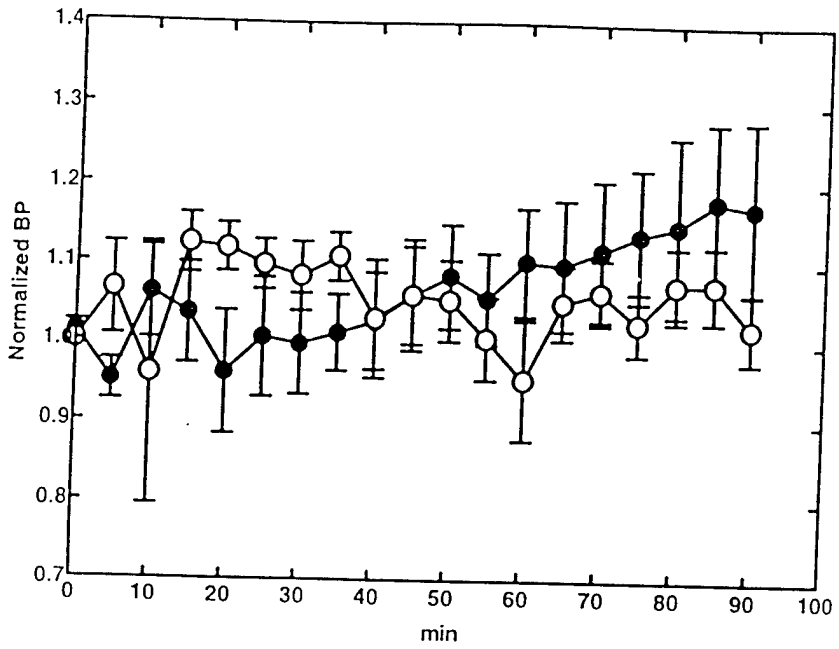

FIG. 1. Mean arterial blood pressure measurements following fluid percussion injury in rats treated with drug (closed circle) or carrier only (open circles). The X axis represents the time following injury in minutes. The Y axis is mean arterial blood pressure in mm Hg. All values are mean ± SEM.

further superoxide production (Hall et al., 1994). Disruption of plasma membranes by lipid peroxidation results in the release of free fatty acids, including arachidonic acid, which reacts to produce superoxide. Loss of plasma membrane integrity sets off a series of events that may lead to superoxide production indirectly through increased calcium influx, increased purine metabolism, interruption of oxidative phosphorylation, recruitment of neutrophils and other immunocytes, and other possible mechanisms (Siesjö et al., 1989; Hall et al., 1994).

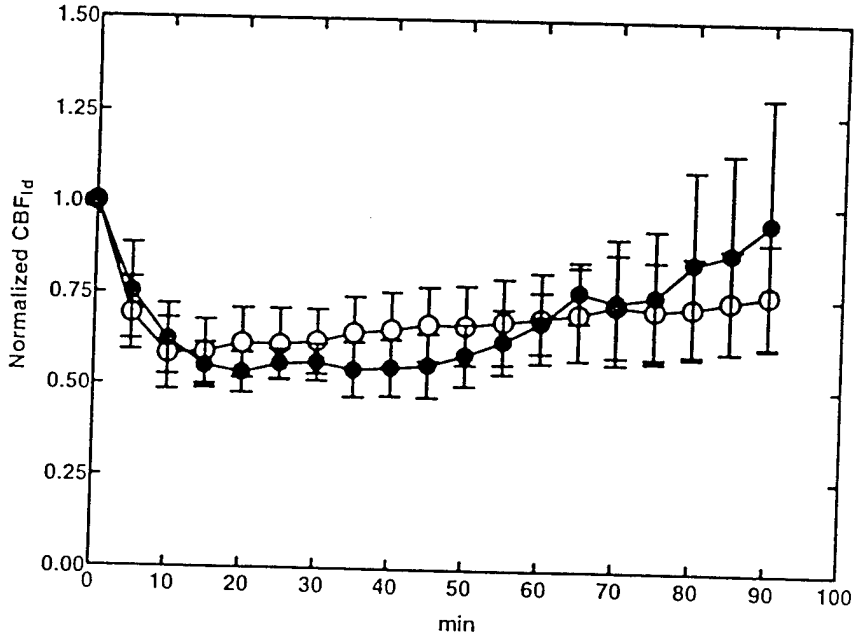

FIG. 2. Normalized cerebral blood flow (CBF) as determined by laser Doppler flowmetry in drug (closed circles and carrier (open circles) treated rats. Time is in minutes following fluid percussion traumatic brain injury (TBI). All values are mean ± SEM.

The drug used in these studies, U-74389G, has been shown to have neuroprotective properties in a stroke model (Muller et al., 1995) and in radiation injury to the brain (Buatti et al., 1996). It also inhibits lipid peroxidation in cultured endothelial cells (Shi et al., 1995) and is protective in other models of tissue injury, such as in the lung (Antonini et al., 1995; Remmers et al., 1996), the heart (Duguay et al., 1996), and skeletal muscle reperfusion injury (Hoballah et al., 1995).

Tirilazad has been shown to reduce reductions in tissue perfusion in various injury models, such as postischemic hypoperfusion in cerebral ischemia (Hall and Yonkers, 1988), posttraumatic tissue hypoperfusion in spinal cord trauma (Hall et al., 1989), hypoperfusion with cortical spreading depression (Hall and Smith, 1991), and hypoperfusion due to cerebral hypoglycemic injury (Maruki, 1995). An increase in tissue perfusion may ameliorate many of the effects of trauma, including the production of superoxide. Conversely, the production of superoxide may produce vasoconstriction through, among other things, the reaction of superoxide with nitric oxide, which may reduce nitric oxide concentration in tissue (Beckman, 1991; Murohara et al., 1993; White et al., 1994). Thus, free radical scavengers may affect vessel tone by reducing superoxide concentrations—activity that has been posited to be related to effects on vascular tone in various models of tissue injury (Beckman, 1991; Murohara et al., 1993).

However, our study fails to demonstrate an effect on postinjury hypoperfusion for U-74389G in this study of fluid percussion cerebral injury despite a reduction of superoxide concentration, which suggests that the effect on tissue perfusion of other lazaroids can be disassociated from the effect on superoxide concentrations. Inconsistent effects on CBF may ultimately relate to limitations in neuroprotection *in vivo* if increases in CBF are necessary in certain models for improvement in outcome. It is possible that with different doses of U-74389G an effect on CBF would appear. Other studies have shown that 21-aminosteroids do not always ameliorate cerebral hypoperfusion (Duckrow and Beard, 1992). It is possible that we may have observed improvements in CBF using different doses of U-74389G. This would not change the main conclusion of this study, which is that reductions in CBF and superoxide anion levels can be disassociated.

Comparison of levels of superoxide reacting to the electrode surface with concentration of superoxide over the pial surface as measured by nitroblue tetrazolium in brain trauma reveals that the reduction rates of NBT and immobilized cytochrome *c* are similar: 50 pmol · min^{-1}

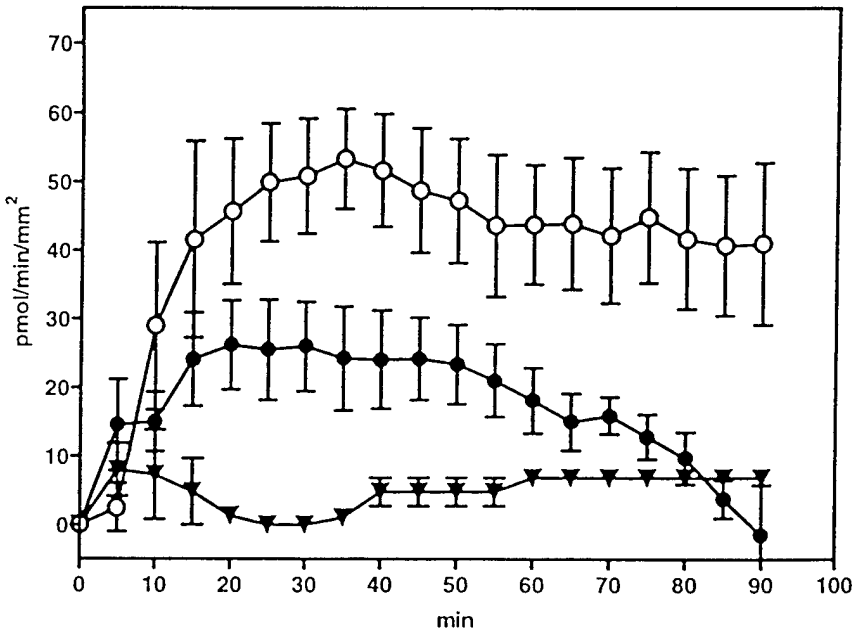

FIG. 3. Cytochrome *c* reduction rates at the electrode surface over the cerebral cortex following fluid percussion injury in rats treated with drug (closed circles) and carrier only (open circles). Measurements of current from bovine serum albumin (BSA)-coated electrode are included for comparison (triangles). Time is in minutes following fluid percussion traumatic brain injury (TBI). Reduction rates are measured relative to the baseline. There is a significant reduction of superoxide levels in drug treated vs. carrier treated rats ($p < 0.001$). Current from BSA-coated electrodes does not change significantly from baseline. All values are mean ± SEM.

· mm^{-2} vs. 41 pmol · min^{-1} · mm^{-2} (Kontos and Wei, 1986). About 20% of the superoxide generated by the reaction of xanthine and xanthine oxidase in solution reacts with cytochrome *c*, either immobilized or free in solution, and a similar proportion of NBT reacts with superoxide generated in aqueous solution (McNeil et al., 1989; McNeil et al., 1992). The remaining 80% of superoxide presumably spontaneously dismutates to form hydrogen peroxide, which does not react with cytochrome *c*.

One disadvantage of using an electrode to detect superoxide anion is that the electrode must be very close to the source of superoxide anion, which is capable of diffusing only a fraction of a millimeter before spontaneously dismutating. Although superoxide anion is capable of crossing membrane barriers and may have originated in the cerebral parenchyma (Kontos et al., 1985), it is also possible that the primary source of the superoxide anion in this study was the pia or the pial vasculature (Kontos et al., 1992). The amount of superoxide interacting with the electrode is highly dependent upon the distance from the source of superoxide to the electrode surface. Although electrode placement was uniform between animals in this study, the inability to know the distance between the source of superoxide and the electrode with precision, among other things, makes it impossible to determine actual superoxide anion production levels with the electrode other than on a relative basis. It is nevertheless valid to correlate superoxide anion concentration changes with CBF as measured by LDF, even though the probes are in slightly different locations, since CBF reductions after TBI tend to be widespread (Yuan et al., 1988; Muir et al., 1992). Central and lateral fluid percussion TBI produces widespread decreases in CBF in rats. After moderate central fluid percussion injury in rats, CBF decreased similarly in the cerebral hemispheres and in the brain stem and cerebellum. Moderate lateral fluid percussion TBI produced significant decreases in brain stem, cerebellar, dienchephalic, and frontal and parietal cortical blood flow on both (i.e., injured and uninjured) sides of the rodent brain (Yuan et al., 1988; Yamakami and McIntosh, 1991). Therefore, although laser Doppler flowmetry measures perfusion in a small area of the cerebral hemispheres, CBF in the cerebral hemispheres seem to reflect global changes in CBF that occur after fluid percussion TBI.

In summary, U-74389G at a dose of 3 mg/kg reduces superoxide concentration without significantly affecting CBF. The treatment dose chosen or the time of administration may, at least in part, explain the difference observed between the effects of this 21-aminosteroid and those of similar agents in similar models observed by other investigators. In any case, our results suggest that the reduction of superoxide anion levels by U-74389G are not due to a mechanism mediated through increased CBF—a finding that may have implications for its mechanisms of neuroprotection.

ACKNOWLEDGMENTS

The authors are grateful for the excellent technical assistance of Charmaine Rea and Kimberly Miller. This work was sponsored by the Upjohn company, Kalamazoo, Michigan, USA, and by grants from the NIH (NS 19355) and the Department of the Army (DAMD17-7008).

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JOURNAL OF NEUROTRAUMA
Volume 15, Number 8, 1998
Mary Ann Liebert, Inc.

Effects of Moderate, Central Fluid Percussion Traumatic Brain Injury on Nitric Oxide Synthase Activity in Rats

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ABSTRACT

Experimental traumatic brain injury (TBI) damages cerebral vascular endothelium and reduces cerebral blood flow (CBF). The nitric oxide synthase (NOS) substrate, L-arginine, prevents CBF reductions after TBI, but the mechanism is not known. This study examined the possibility that posttraumatic hypoperfusion is due to reductions in the substrate sensitivity of NOS which are overcome by L-arginine. Isoflurane-anesthetized rats were prepared for TBI (midline fluid-percussion, 2.2 atm), sham-TBI, or no surgery (control), and were decapitated 30 min after injury or sham injury. The brains were removed and homogenized or minced for measurements of crude soluble or cell-dependent stimulated NOS activity, respectively. Baseline arterial oxygen, carbon dioxide, pH, or hemoglobin levels did not differ among control, sham, or TBI groups. Total cortical soluble NOS activity in TBI-treated rats was not significantly different from either untreated or sham groups when 0.45 μM or 1.5 μM L-arginine was added. Also, there were no differences in cell-dependent NOS activity among the three groups stimulated by 300 μM *N*-methyl-D-aspartate, 50 mM K^+, or 10 μM ionomycin. These data suggest that TBI reduces CBF by a mechanism other than altering the substrate specificity or activation of nNOS.

Key words: arginine; calcium; cerebral blood flow; cortex; fluid percussion; glutamate

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) results in reduced cerebral blood flow (CBF) in the first few hours after injury (Bouma et al., 1991, 1992). Although the role of posttraumatic hypoperfusion in pathophysiology is not known, evidence of ischemia in most TBI patients (Graham et al., 1978) suggests that CBF reductions may be important contributors. Early posttraumatic hypoperfusion occurs after experimental TBI (Yuan et al., 1988; Yamakami and McIntosh, 1989, 1991). The causes of significant reductions in CBF after TBI in patients or experimental animals are not known but posttraumatic hypoperfusion may result from impairment or destruction of a cerebral vasodilatory mechanism. The endothelium-dependent relaxing factor, nitric oxide (NO), is one such cerebral vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). Evidence that inhibition of NO synthesis decreases CBF (Beckman et al., 1991; Tanaka et al., 1991; DeWitt et al., 1992; Pelligrino et al., 1993) suggests a resting cerebral vasodilatory tone owing to the continuous production of NO. Nitric oxide is a free radical that is inactivated (Rubanyi and Vanhoutte, 1986) or converted to an even more reactive species such as the peroxynitrite anion (Beckman, 1991; Beckman et al., 1994; Crow and Beckman, 1995) by oxygen free radicals

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such as superoxide. Superoxide anion radicals are produced by fluid-percussion TBI (Wei et al., 1981; Fabian et al., 1995), perhaps as a byproduct of trauma-induced increases in prostaglandin synthesis (DeWitt et al., 1988). Free radicals contribute to the pathophysiology of TBI because the cyclooxygenase inhibitor indomethacin or the free radical scavenger superoxide dismutase reduces impaired cerebral vascular reactivity and endothelial damage after TBI (Wei et al., 1981). Recent evidence that treatment with L-arginine or superoxide dismutase improves CBF after experimental TBI (DeWitt et al., 1997) supports the hypothesis that radical-mediated inactivation of NO or nitric oxide synthase (NOS) contributes to posttraumatic hypoperfusion. Whether TBI affects NO levels directly or reduces NO by affecting NOS remains unclear. To determine whether NOS activity is altered by brain trauma, the conversion of L-arginine to citrulline was measured in rats subjected to moderate central fluid-percussion TBI.

MATERIALS AND METHODS

Surgical Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2/room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline fluid-percussion TBI as previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagitally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma and a modified Luer-Lok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acylic. Isoflurane was decreased to 1.5%; the rats were connected to the trauma device and subjected to moderate (2.2 atm) TBI. The animals in the sham group were subject to the same surgical procedure as the TBI group with the exception of the actual insult. Brains were harvested 30 min after surgery (control group did not have surgery), TBI, or sham-TBI, and the cortices were used for cell-independent and cell-dependent biochemical assays as described below. All assays were performed blinded as to experimental group.

Experimental Design

All rats were prepared for sham injury or fluid-percussion TBI as described above. Control rats were decapitated without surgical preparation. To measure total baseline soluble NOS activity, control rats (n = 10), or rats subjected to sham injury (n = 5) or moderate (2.2 atm), central, fluid percussion TBI (n = 5) were decapitated approximately 30 min after injury or sham-injury. Brains were removed, the hemispheres were separated from the brain stem and prepared for crude enzyme assessments of total soluble NOS activity as described below. Saturating concentrations of cofactors and sufficient amounts of substrate were combined with NOS inhibitors when appropriate. The inhibitors used in these experiments were 3-bromo-7-nitroindazole (30 μM) and *S*-methyl-thiocitrulline (10 μM).

To measure cell-dependent NOS activity, control rats (n = 9) or rats subjected to sham-injury (n = 5) or moderate (2.2 atm) central fluid-percussion TBI (n = 5) were decapitated approximately 30 min after injury or sham injury. Brains were removed and mince preparations were prepared for assessment of NOS activity in response to stimulation with 300 μM NMDA, 50 mM potassium chloride, or 10 μM ionomycin.

In both studies, statistical differences were determined by analysis of variance followed by Dennett's test where appropriate. A p value of <0.05 was considered significant.

Cell-Independent NOS Activity

Measurements of soluble NOS activity in crude enzyme preparations based on methods of Bredt and Snyder (1989) were made by obtaining and homogenizing the cerebral cortex in three volumes buffer containing 0.32 M sucrose, 20 mM HEPES, 0.5 mM EDTA, and 1 mM dithiothretol. This homogenate was centrifuged for 5 min at 3,000*g*. The supernatant was spun at 20,000*g* for 15 min. The supernatant from the second spin was passed over a Dowex AG50WX-8 ion exchange column to remove the endogenous arginine. Activity of the soluble NOS enzyme was monitored by adding Ca^{2+} (0.9 mM), NADPH (10 mM), and [^{3}H]arginine (50 mM) (Amersham, Arlington Heights, IL) to the homogenate and measuring the levels of [^{3}H]citrulline produced in a 20-min incubation at room temperature. In a group of rats, the NOS inhibitors 3-bromo-7-nitroindazole or *S*-methyl-thiocitrulline were added during this incubation period. The [^{3}H]arginine was separated from the [^{3}H]citrulline by ion exchange chromatography as described be

low. These data are presented as amount of [^{3}H]citrulline divided by the amount of [^{3}H]arginine + [^{3}H]citrulline × 100/mg of protein.

Cell-Dependent NOS Activity

Cell-dependent, stimulated NOS activity in cortical minces was also determined by a modification of the method of Bredt and Snyder (1989). The animals were killed by decapitation and their brains removed and placed into ice-cold, oxygenated (95% O_2, 5% CO_2) modified Krebs bicarbonate buffer containing 0.3 mM $CaCl_2$, 118 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.2 mM glucose, and 1.2 mM $MgSO_4$. The cortex was dissected and cross-chopped at 450 μm on a McIlwain tissue chopper. The minces were transferred to fresh ice-cold buffer and incubated on ice for 15 min. The buffer was changed and the minces were incubated at 37°C for 1 h with two additional buffer changes.

The minces were then transferred to a conical bottom tube on ice for gravity packing. Minivials were prepared that contained 30-μl aliquots of the appropriate drug. Freshly oxygenated buffer (270 μl) containing 30 nM L-[^{3}H]arginine (Amersham) was added. Fifty microliters of the gravity-packed slices were added in rapid succession to the minivials and incubated for 5 min. The reaction was stopped with 700 μl of an ice-cold solution of 4 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM unlabeled L-arginine dissolved in buffer. The minivials were then centrifuged at 1,000*g* and the supernatant decanted. The pellet was sonicated in 1 ml of 1 M trichloracetic acid (TCA). The vials were then spun at 12,000*g*, and an aliquot of the supernatant was collected and extracted three times with two volumes of ethyl ether to remove the TCA. The ether was allowed to evaporate overnight and a 0.5-ml aliquot of each sample was passed through 2 ml of a sodium equilibrated ion exchange resin (Dowex AG50W-X8) to separate the L-[^{3}H]citrulline formed from L-[^{3}H]arginine. L-[^{3}H]Citrulline was eluted in 4 ml of HEPES and combined with the sample effluent. L-[^{3}H]Arginine was eluted in 6 ml of HEPES at pH 12. This procedure eluted 98% of the total added radioactivity. Cell-dependent NOS activity is presented as [^{3}H]citrulline/[^{3}H]arginine + [^{3}H]citrulline × 100 minus a boiled tissue blank value divided by percent conversion of the same day untreated control.

RESULTS

All values in the text, tables, and figures are means ± standard error of the mean. Baseline mean arterial pressure (MAP) in the rats prepared for measurement of cell-dependent NOS activity in mince preparations was significantly higher than baseline MAP in the rats prepared for the crude soluble enzyme assay; however, there were no significant differences in MAP between the sham-injured and the injured groups. There were no differences among the groups in baseline (pretrauma) arterial oxygen, carbon dioxide, pH, or hemoglobin levels (Table 1). Levels of fluid-percussion TBI (2.2 atm, 22 ms) and MAP during transient arterial hypertension in response to TBI were the same in all groups (Table 1).

Total cortical soluble NOS activity was measured in control (n = 10), sham-injured (n = 5), or moderate TBI (n = 5) rats with either 0.45 or 1.5 μM added exogenous arginine (Fig. 1). There were no significant differences in soluble NOS activity between control, sham, or TBI groups at either concentration of added arginine. L-Nitroarginine methyl ester (L-NAME) blocked 97% of NOS activity when used to perfuse the brain during the surgery (25 mg/kg i.v.) or when added during the enzyme assay (30 μM). 3-Bromo-7-nitroindazole (30 μM) and *S*-methyl-thiocitrulline (10 μM), more specific inhibitors of the neuronal isoform of NOS, blocked 96% and 99% of NOS activity, respectively, when used during the enzyme assay (unpublished observations).

We have previously shown that 5-min incubation with 300 μM NMDA, 10 μM ionomycin or 50 mM KCl are able to significantly stimulate NOS activity in the cell-intact preparation each via different mechanisms (Ala-

TABLE 1. MEAN ARTERIAL BLOOD PRESSURE (MAP, mm Hg) AND ARTERIAL BLOOD GAS VALUES (mm Hg) AND HEMOGLOBIN (Hgb, g/dl) LEVELS IN RATS PREPARED FOR MEASUREMENT OF BASELINE OR STIMULATED NOS ACTIVITY AFTER MODERATE FLUID PERCUSSION TBI OR SHAM TBI

Experiment	*Group*	*MAP baseline*	*MAP peak*	*Arterial blood*			
				pH	*pO_2*	*pCO_2*	*Hgb*
Crude enzyme preparation	Sham	104.2 ± 1.4	-	7.38 ± 0.01	300 ± 16	40 ± 1	12.3 ± 0.2
	TBI	103.5 ± 1.7	140.7 ± 5.7	7.37 ± 0.01	303 ± 23	38 ± 1	12.8 ± 0.3
Mince preparation	Sham	121.3 ± 2.0	-	7.40 ± 0.01	311 ± 18	38 ± 1	12.3 ± 0.2
	TBI	126.5 ± 2.0	155.8 ± 7.6	7.40 ± 0.01	303 ± 13	37 ± 1	13.2 ± 0.2

Data are mean ± SEM

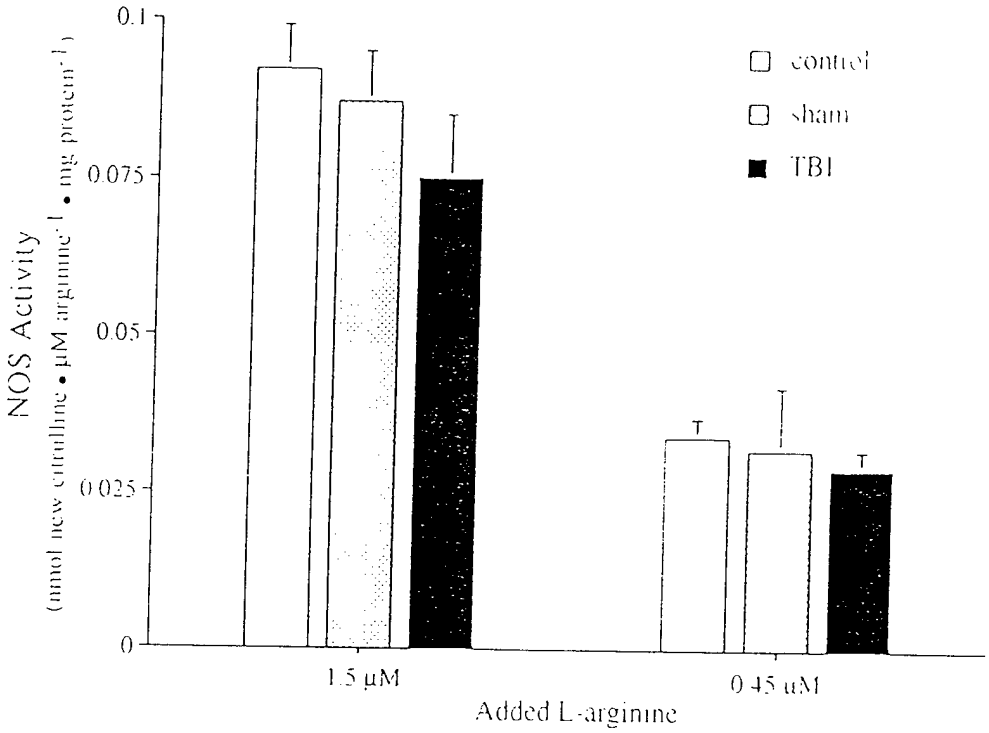

FIG. 1. Total baseline soluble nitric oxide synthase (NOS) activity determined from crude enzyme preparations from control (unoperated, $n = 10$) rats and rats subjected to sham injury ($n = 5$) or moderate ($n = 5$) fluid percussion traumatic brain injury (TBI).

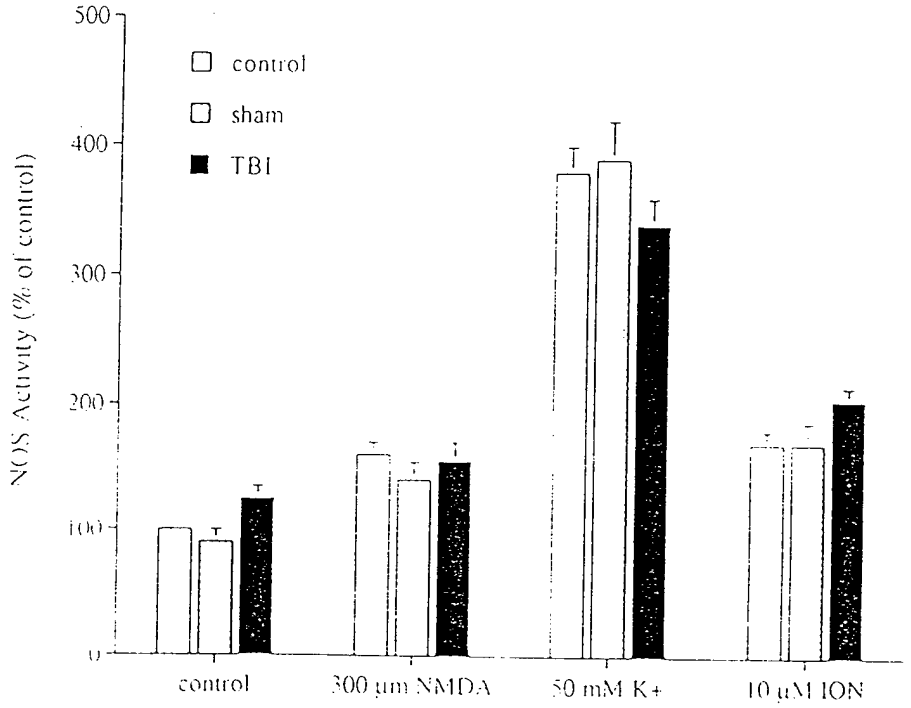

FIG. 2. Stimulated cell-dependent nitric oxide synthase activity (NOS) in mince preparations from control (unoperated, $n = 9$) rats and rats subjected to sham injury ($n = 5$) or moderate ($n = 5$) fluid-percussion traumatic brain injury (TBI) and then exposed to *N*-methyl-D-aspartate (NMDA), potassium chloride (K^+), or ionomycin (ION). Cit, citrulline; Arg, arginine.

garsamy et al., 1994). Since it is possible that cell-dependent NOS activity may be changed following TBI without affecting the total crude soluble activity, we compared baseline activity and NOS activity in response to the above stimuli. NOS activity in control (n = 9), sham injury (n = 5), and moderate TBI (n = 5) were not significantly different at either baseline or stimulated conditions (Fig. 2).

DISCUSSION

These studies demonstrated that moderate central fluid-percussion TBI did not significantly reduce basal soluble NOS activity or cell-dependent NOS activity in response to stimulation with the glutamate receptor agonist NMDA, to depolarization by KCl, or to a calcium ionophore (ionomycin).

Reduced CBF after TBI occurs in humans (Bouma et al., 1991; Kobayashi et al., 1991) as well as in experimental animals (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1988; Armstead and Kurth, 1994). One possible mechanism by which this could occur is a reduction in the amount of NO, the major endothelium-derived relaxing factor (Ignarro et al., 1987), which is generated and released. Because the amount of NO that is released is directly proportional to the activity of NOS, it is possible that reduction of NOS activity would lead to a reduction in NO-mediated vasodilation. Reduction of NOS activity could be the result of enzyme destruction or an alteration in substrate or cofactor dependence after injury. Studies showing increased CBF after infusion of L-arginine, the NOS substrate, after TBI (DeWitt et al., 1997) suggest that changes in substrate availability may be the mechanism of reduced CBF. Our experiments failed to show any significant changes in total soluble NOS activity at either concentration of added substrate, suggesting that neither the amount of NOS nor its affinity for arginine was affected by moderate TBI.

This study was intended to test the hypothesis that posttraumatic hypoperfusion is due to reductions in NOS activity. rats were killed 30 min after injury or sham injury. Thirty minutes after moderate TBI is a time point when CBF is consistently reduced (Yuan et al., 1988; Yamakami et al., 1989; Muir et al., 1992; Dietrich et al., 1996). CBF returns to preinjury levels 2–4 h after TBI. In addition, hemorrhagic hypotension results in significant reductions in NOS activity 20 min after resuscitation (Kovach et al., 1994) and plasma nitrate/nitrite levels increase significantly 30 min after reperfusion after middle cerebral artery occlusion (Kimura et al., 1994). These studies indicate that NOS activity and NO metabolism change rapidly after cerebral injury, within the time period during which significant reductions in CBF occur after TBI.

Additionally, it is possible that NOS cofactor dependence or other changes related to calcium-mediated activation could lead to decreased NO synthesis. In this case, there would be a smaller response to drugs that stimulate NOS. We have previously shown that NMDA, K^+, and a calcium ionophore, ionomycin, each stimulate NOS activity by different calcium-dependent mechanisms (Alagarsamy et al., 1994). However, TBI had no effect on either basal or stimulated NOS activity in the cortex. These data suggest that TBI does not affect the ability of NOS to synthesize NO under a variety of conditions.

An alternate explanation is that the during preparation of the tissue, either during the surgery itself or during the preparation of tissue for the enzymatic assay, there was some artifact-induced reduction in baseline NOS activity. In this case, a further reduction in NOS activity due to TBI may not have been detected. It is possible that the isoflurane used during the surgical procedure may have significantly inhibited NOS activity (Terasako et al., 1994; Tobin et al., 1994). However, because the anesthetic was withdrawn 30 min prior to decapitation, the residual amount present in the *in vitro* preparations would have been minimal (Berg-Johnsen and Langmoen, 1987). Additionally, the levels of enzyme activity in both the crude enzyme preparation and the mince preparation were similar in the untreated animals and the sham surgery controls, suggesting that the isoflurane did not significantly inhibit NOS in our test preparations.

Although previous studies suggest that changes in substrate availability are a likely mechanism by which NOS activity might be altered (Morikawa et al., 1994; Fabricius et al., 1995), it is possible that sensitivity to the other cofactors necessary for NOS activation may be changed. Also, although the affinity of NOS for arginine may not be affected, it is possible that the availability of arginine is changed as a result of TBI. It is possible that TBI causes a reduction in the transport of arginine into the cells, thereby making arginine a limiting factor. Therefore, adding exogenous arginine may increase CBF after TBI (DeWitt et al., 1997) by helping to overcome reductions in transport efficiency. However, our experiments suggest that this may not be the case since, in the mince preparation, there were no differences in the total [^{3}H]arginine uptake in the various groups (data not shown).

Another possibility is that there are significant changes in NOS activity that are localized to discreet areas immediately surrounding the injury site. After central fluid-percussion injury, hypoperfusion occurs throughout the cerebral hemispheres (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1988). However, other studies indicate that lateral fluid percussion TBI produces focal as well

as more widespread cerebral hypoperfusion in rats (Dietrich et al., 1996). Because we used the total cortex for our analyses, it is possible that reductions in NOS activity that occurred in small regions were diluted by surrounding areas in which NOS activity remained unchanged after TBI.

Brain temperature was not measured or controlled in this study. Although it is possible that brain temperature decreased after TBI, thereby protecting NOS from TBI-related damage, previous studies have reported on differences in brain temperature between fluid percussion TBI and sham injury in anesthetized rats (Jiang et al., 1991). However, TBI-induced brain temperature decreases don't prevent posttraumatic cerebral blood flow (CBF) decreases since previous studies have demonstrated that posttraumatic hypoperfusion occurs even if brain temperature is not controlled after TBI (Yuan et al., 1988; Yamakami et al., 1989; Muir et al., 1992). The important observation is that global NOS activity did not decrease 30 min after TBI, a time that is associated with significant reductions in CBF.

Although our data suggest that TBI does not affect total or stimulated NOS activity, it is still possible that there may be changes in NOS after TBI that were not detected. Although most NOS activity was blocked by the specific nNOS inhibitor, there was some residual activity. It is possible that this residual activity represented the pool of NOS that is sensitive to TBI. However, experiments attempting to identify changes in the residual pool were unsuccessful as the remaining activity was below the sensitivity limits of our assay.

These studies demonstrated that moderate central TBI does not affect basal or stimulated NOS activity. Although moderate TBI had no measurable effect on NOS activity, it is possible that a more severe TBI could have produced significant changes. Our present observations that NOS activity is unaffected by TBI, coupled with previous evidence that L-arginine improves CBF after TBI (DeWitt et al., 1997), suggest that TBI decreases NO levels by destroying NO directly, rather than by affecting NO production. This hypothesis is supported by evidence that TBI produces the superoxide anion radical (Wei et al., 1981; Fabian et al., 1995), which inactivates NO (Rubanyi and Vanhoutte, 1986) or rapidly converts it to the powerful oxidant, peroxynitrite (Beckman, 1991, 1994). Further studies involving direct measurements of NO levels after TBI are required to determine the effects of TBI on NO within the central nervous system and the cerebral vasculature.

ACKNOWLEDGMENTS

We thank Christopher Tidwell for technical expertise, Faith McLellan for editorial assistance, and Deborah Robb and Lori Spurger for help with organizing and typing this manuscript. We are grateful to Donald S. Prough, M.D., and our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. This work was supported, in part, by NS19355 (NIH/NINDS) and DAMD17-97-1-7008 (Department of the Army).

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JOURNAL OF NEUROTRAUMA
Volume 16, Number 12, 1999
Mary Ann Liebert, Inc.

Traumatic Brain Injury Reduces Myogenic Responses in Pressurized Rodent Middle Cerebral Arteries

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ABSTRACT

Traumatic brain injury (TBI) reduces cerebral vascular pressure autoregulation in experimental animals and in patients. In order to understand better the mechanisms of impaired autoregulation, we measured myogenic responses to changes in intraluminal pressure *in vitro* in pressurized, rodent middle cerebral arteries (MCAs) harvested after TBI. In an approved study, male Sprague-Dawley rats (275–400 g) were anesthetized, intubated, ventilated with 2.0% isoflurane in O_2/air, and prepared for fluid percussion TBI. The isoflurane concentration was reduced to 1.5%, and rats ($n = 6$ per group) were randomly assigned to receive sham TBI followed by decapitation 5 or 30 min later or moderate TBI (2.0 atm) followed by decapitation 5 or 30 min later. After decapitation, MCA segments were removed, mounted on an arteriograph, and pressurized. MCA diameters were measured as transmural pressure was sequentially reduced. MCA diameters remained constant or increased in the sham groups as intraluminal pressure was reduced from 100 to 40 mm Hg. In both TBI groups, diameter decreased with each reduction in pressure. In summary, MCAs removed from uninjured, isoflurane-anesthetized rats had normal vasodilatory responses to decreased intraluminal pressure. In contrast, after TBI, myogenic vasodilatory responses were significantly reduced within 5 min of TBI and the impaired myogenic responses persisted for at least 30 min after TBI.

Key words: autoregulation; cerebral arteries; myogenic response; traumatic brain injury

INTRODUCTION

IN 1890, ROY AND SHERRINGTON stated that "the brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of functional activity." ~~He~~ THEY recognized that the cerebral circulation was capable of reacting to changes in systemic physiology in order to minimize changes in the brain's external environment. Forty-seven years after the landmark work of Roy and Sherrington, Fog (1937) reported that the pial circulation *in vivo* is capable of significant reductions in cerebral vascular resistance during reductions in systemic arterial blood pressure. Lassen (1959) further defined the concept of autoregulation in the cerebral circulation.

The mechanisms that contribute to this phenomenon, termed "pressure autoregulation," while not fully understood, are believed to be due to metabolic, neurogenic,

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or myogenic mechanisms. Proposed metabolic mechanisms underlying autoregulation include changes in perivascular pH, K^+ concentration, and O_2 tension, but since these hypotheses have proven problematic (Paulson et al., 1990; Busija, 1993), the metabolic products that mediate autoregulatory vasodilation remain unknown. The cerebral circulation is surrounded by a dense plexus of perivascular nerves containing, among others, vasodilatory neurotransmitters such as calcitonin gene–related peptide (CGRP; Burnstock and Ralevic, 1994; Branston, 1995). Evidence that autoregulation is impaired *in vivo* in rats by antibodies to CGRP or capsaicin, which depletes transmitters from perivascular sensory nerves, suggests that neurogenic mechanisms contribute to autoregulation (Hong et al., 1994). In addition, the cerebral vasculature, as well as other vascular beds, is capable of intrinsic vasodilation or vasoconstriction in response to changes in transmural (intramural) pressure *in vitro*. Meininger and Davis (1992) defined spontaneous or intrinsic tone (i.e., myogenic tone) as a maintained state of partial smooth muscle activation and defined the myogenic response *in vitro* separately as a contraction of a blood vessel in response to increases in transmural pressure. Direct myogenic responses to changes in transmural pressure *in vitro* occur in the cerebral and other vascular beds (Osol et al., 1989; McCarron et al., 1989; Meininger and Davis, 1992; Bayliss, 1902). In the cerebral circulation, myogenic tone and contraction in response to increases in intravascular pressure may be due to voltage-dependent Ca^{2+} channels (Knot and Nelson, 1998) or to chloride channels (Nelson et al., 1997) *in vitro*. Numerous mechanisms have been suggested to mediate myogenic tone and response to changes in transmural pressure, but the mechanisms of these responses and their contributions to cerebral autoregulation remain to be determined. It is conceivable that metabolic, neurogenic, and myogenic mechanisms all contribute to autoregulation, acting as redundant systems, or, perhaps, acting at different levels of arterial blood pressure.

CAPITALIZE

In 1974, Overgaard and Tweed reported that TBI results in significant reductions in autoregulatory responses to changes in arterial blood pressure *in vivo*. Subsequent *in vivo* studies have confirmed that TBI reduces autoregulation in young (Muizelaar et al., 1989) and adult humans (Enevoldsen and Jensen, 1978; Bouma and Muizelaar, 1990) even after relatively mild TBI (Jünger et al., 1997). Wei et al. (1980) reported that dilator responses to hypotension in feline pial arteries are abolished by TBI. Using H_2 clearance and radioactive microsphere methods, respectively, Lewelt et al. (1980) and DeWitt et al. (1992) demonstrated impaired CBF autoregulation in response to arterial hypotension after TBI. Because the mechanisms that contribute to normal autoregulation are unclear, the mechanisms of traumatic damage to autoregulation also are not known.

Studies of the effects of TBI on the cerebral vasculature *in vivo* following TBI are complicated by the need to differentiate the direct effects of TBI on cerebral vessels from indirect effects that may be mediated by substances that may be released from the traumatized brain. Trauma produces marked increases in vasoactive prostaglandins (DeWitt et al., 1988; Ellis et al., 1981) and oxygen free radicals (Fabian et al., 1995; Wei et al., 1981), substances which may be produced by brain parenchyma but influence the cerebral vasculature. This concept is supported by recent *in vitro* evidence that arterial ring segments isolated from the rodent middle cerebral artery (MCA) after TBI respond normally to acetylcholine (ACh; Bukoski et al., 1997), while pial arteries and arterioles *in vivo* do not exhibit normal vasodilatory response to acetylcholine (Ellison et al., 1989; Kontos and Wei, 1992). In addition, recent evidence (Golding et al., 1998) indicates that myogenic responses are reduced *in vitro* in cerebral arteries harvested from rats 24 h after severe cortical impact injury. In order to determine whether cerebral arterial vasodilatory responses to reductions in transmural pressure are affected by TBI, we studied myogenic responses to hypotension in pressurized MCA segments harvested from rats after moderate, central fluid percussion TBI.

ACh contrast

METHODS

Surgical Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2/room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed in a femoral artery and vein for arterial pressure monitoring and for drug infusion, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline fluid percussion TBI as previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma, and a modified LuerLok syringe hub was placed over the exposed dura and bonded in place with cyano-

acrylic adhesive and covered with dental acrylic. Isoflurane was lowered to 1.5%, and the rats connected to the trauma device and randomly assigned to one of the groups listed below.

Measurement of Middle Cerebral Arterial Diameter

After TBI or sham injury, anesthetized rats were decapitated, the brains were removed, and the MCAs were harvested. The tissue around the MCAs was cut with microfine scissors. The MCAs were gently reflected, beginning at the circle of Willis and continuing dorsally for 4–5 mm. The connective tissue was gently removed from the MCAs, and the vessels were mounted in an arteriograph as described in detail (Bryan et al., 1996). The proximal end of the vessels were cannulated with a glass micropipette and secured with a 10-0 nylon suture. The lumina were gently perfused with physiologic salt solution (PSS) to remove residual blood. The distal ends of the vessels were cannulated with a second micropipette and advanced until the desired location (a segment without a leak) was reached. After securing the distal end with the suture, the longitudinal slack was removed by increasing the separation between the two pipettes. The vessels were pressurized to 50 mm Hg by raising a reservoir containing PSS and connected to the pipettes by tygon tubing. The mounted arterial segments were bathed in PSS of the following composition (NaCl, 130 mM; KCl, 4.7 mM; $MgSO_4 7H_2O$, 1.17 mM; glucose, 5 mM; $CaCl_2$, 1.50 mM, $NaHCO_3$, 15 mM). When gassed with a mixture of 21% O_2, 5% CO_2, and balance N_2, this solution has a pH of 7.4. After mounting, the PSS was warmed from room temperature to 37°C, and the arterial segments were allowed to equilibrate for 60 min with transmural pressure set at 50 mm Hg by raising reservoir bottles connected to the micropipettes. A pressure transducer between the micropipettes and the reservoir bottles was used to monitor transmural pressure within the arterial segment. The vessels were magnified with an inverted microscope equipped with a video camera and a monitor. Arterial diameter was measured using a video scaler calibrated with an optical micrometer. After 1 h of stabilization at an intraluminal pressure of 50 mm Hg, each vessel was exercised to decrease mechanical hysteresis by changing the pressure between 20 and 40 mm Hg three times, allowing 5 min between pressure changes.

At the end of the equilibration period, the transmural pressure was raised to 100 mm Hg. Myogenic responses were tested by decreasing luminal pressure in increments of 20 mm Hg with a 10-min equilibration period at each pressure level before diameter measurements are made. After the initial myogenic responses were evaluated, the PSS was replaced with Ca^{2+}-free PSS and myogenic responses were assessed again.

Experimental Design

Rats were randomly assigned to one of the following four groups ($n = 6$ rats per group): sham-5, MCA were harvested 5 min after sham injury; sham-30, MCA were harvested 30 min after sham injury; TBI-5, MCA were harvested 5 min after moderate (2.0 atm) central TBI; and TBI-30, MCA were harvested 30 min after moderate (2.0 atm) central TBI.

Data Analysis

The myogenic response to changes in transmural pressure was assessed by calculating a myogenic response index (MRI) for each level of transmural pressure, as described elsewhere (Davis, 1993; Golding et al., 1998):

$$MRI = 100 \cdot \left\{ \frac{(D_f - D_i)}{D_i} \right\} \cdot \left\{ \frac{1}{P_f - P_i} \right\}$$

where D_f is the final inner diameter, D_i is the initial inner diameter, P_f is the final transmural pressure, and P_i is the initial pressure.

Baseline (100 mm Hg) MCA diameters were compared between the combined sham-injured and TBI groups using a Wilcoxon two-sample test. Arterial inner diameter data during progressive reductions in transmural pressure were expressed in μm or were normalized to a percent of baseline diameters and analyzed using analysis of variance for a two-factor experiment with repeated measures on time. The two factors were treatment group (sham or TBI) and transmural pressure. The MRI data were similarly analyzed using repeated measures analysis of variance with the two factors of treatment or pressure. The number of treatment groups was four, and the pressure points tested were baseline (100 mm Hg) and 80, 60, 40, and 20 mm Hg. Post hoc analyses were performed using the Student-Newman-Keuls procedure for multiple comparisons of individual points. All data in the text, table, and figures are expressed as means ± standard errors of the means.

RESULTS

Mean arterial blood pressure (MAP) before and 3 min after TBI and arterial pH, pCO_2, pO_2, and hemoglobin concentrations were similar among the four groups (Table 1). Within 30 sec of TBI, mean arterial pressure increased significantly above pre-TBI levels in the 5-min ($p = 0.0002$) and 30-min ($p = 0.0018$) TBI groups

TABLE 1. MEAN ARTERIAL BLOOD PRESSURE AND ARTERIAL BLOOD GASES AT THE TIME OF EUTHANASIA AND MIDDLE CEREBRAL ARTERY HARVEST (EXCEPT MAP_{pre})

	Experimental group			
Variable	*5-min sham*	*30-min sham*	*5-min TBI*	*30-min TBI*
MAP_{pre}	83 ± 3	88 ± 3	85 ± 1	88 ± 3
$MAP_{post\ (30\ sec)}$	84 ± 3	89 ± 2	138 ± 7*	155 ± 10*
$MAP_{post\ (3\ min)}$	83 ± 3	90 ± 3	72 ± 5	81 ± 5
pH	7.48 ± 0.03	7.46 ± 0.02	7.50 ± 0.02	7.46 ± 0.02
PaO_2	264 ± 23	271 ± 29	248 ± 16	301 ± 9
$PaCO_2$	33 ± 2	33 ± 1	31 ± 2	34 ± 2
Hgb	12.9 ± 0.7	13.5 ± 0.5	12.8 ± 0.8	12.9 ± 0.3

MAP_{pre}, mean arterial pressure (mm Hg) before TBI; MAP_{post}, mean arterial pressure (mm Hg) after TBI; PaO_2, partial pressure of arterial oxygen (mm Hg); $PaCO_2$, partial pressure of arterial carbon dioxide (mm Hg); Hgb, arterial hemoglobin concentration (g/dL). Data represented as mean ± SEM.

*$p < 0.05$ compared to MAP_{pre}.

(Table 1). The acute, posttraumatic hypertension was transient, with MAP returning to baseline levels within 2 min of TBI. All MCAs in all four groups developed spontaneous tone as the temperature of the bath was increased to 37°C. Resting (i.e., 100 mm Hg transmural pressure) arterial diameters were larger in the TBI groups than the sham-TBI groups ($p = 0.011$; Table 2).

In both the 5- and 30-min sham-TBI groups, arterial inner diameters did not change or increased as intraluminal pressure was reduced from 100 to 40 mm Hg (Fig. 1). When intraluminal pressures were reduced from 40 to 20 mm Hg, the arterial diameters decreased, although they remained at or above baseline in both sham-TBI groups, even at 20 mm Hg. There were no statistically significant differences in arterial diameters at any intraluminal pressure level between the 5- and 30-min sham-TBI groups.

In both the 5- and 30-min TBI groups, MCA inner arterial diameters decreased sequentially as intraluminal pressure was reduced (Fig. 1). Repeated measures analysis of variance indicated a significant treatment effect at both 5 min ($p = 0.0031$) and 30 min ($p = 0.0007$) as well as a significant treatment versus pressure interactions in both the 5-min ($p = 0.0477$) and 30-min ($p = 0.0035$) treatment groups (Table 3). The changes in inner diameter in arteries harvested 30 min after TBI were slightly greater than inner diameter changes in arteries harvested 5 min after TBI, although the small differences were not statistically significant.

The MRI is the percent change in arterial inner diameter per percent change in transmural pressure. A positive MRI indicates that the transmural pressure change and the arterial diameter change were in the same direction while a negative MRI indicates that pressure and diameter moved in opposite directions (i.e., when pressure was reduced, diameter increased). In the sham-injured rats, MRI was negative as pressure was decreased from 100 to 80, 80 to 60, and 60 to 40 mm Hg (Fig. 2). In contrast, MRI was positive in the 5- and 30-min TBI groups

TABLE 2. INNER DIAMETER MEASUREMENTS (μm) FROM MIDDLE CEREBRAL ARTERIES BEFORE REDUCTIONS IN TRANSMURAL PRESSURE

	Experimental group			
Variable	*5-min sham*	*30-min sham*	*5-min TBI*	*30-min TBI*
Normal	91.3 ± 9.2	95.3 ± 3.7	132.2 ± 21.1*	127.3 ± 9.9*
Ca^{2+}-free	243.3 ± 11.6	245.7 ± 7.4	231.7 ± 15.9	255.2 ± 6.3

Each vessel was measured at a pressure of 100 mm Hg in normal physiological salt solution (PSS) and, at the end of the experiment, in Ca^{2+}-free PSS. Data represented as mean ± SEM.

*$p < 0.05$ compared to sham TBI.

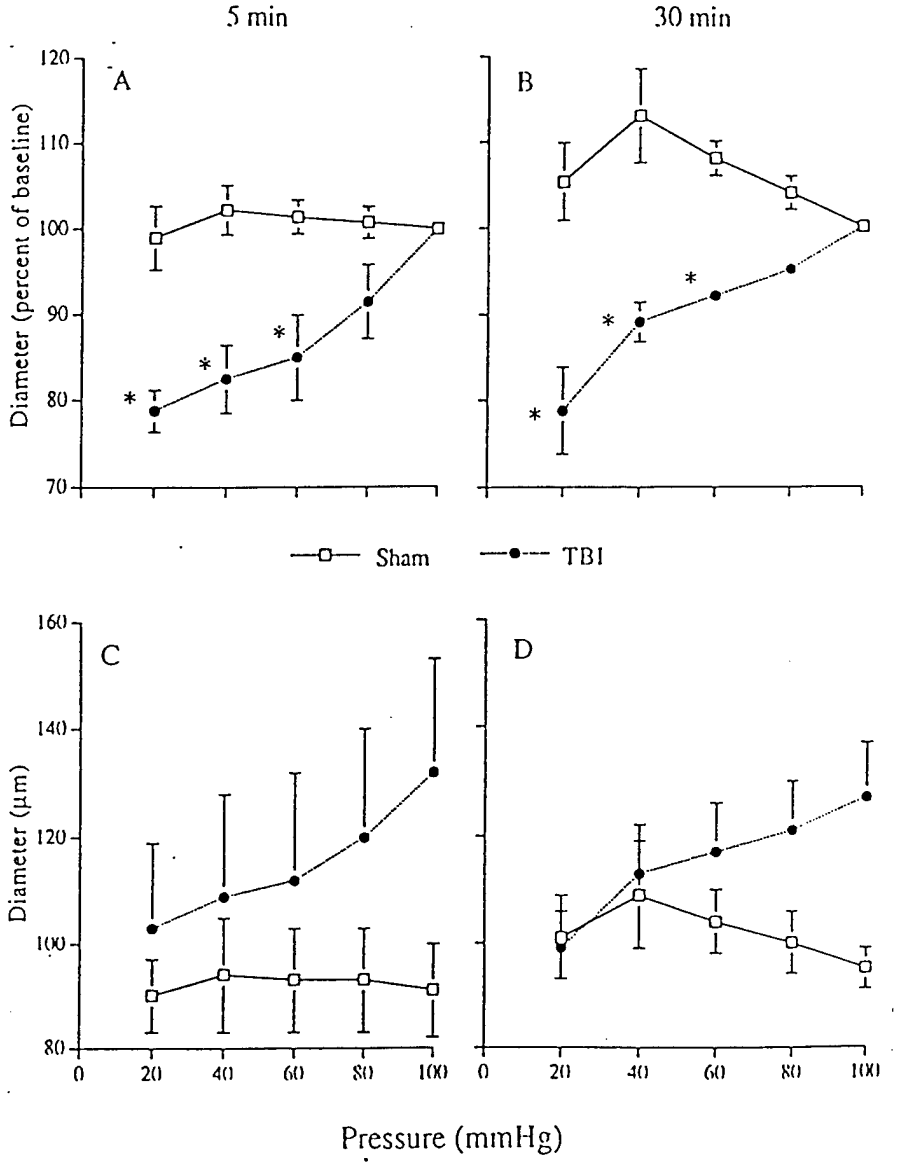

FIG. 1. Diameters of middle cerebral arteries (MCAs) at different transmural pressures. The MCAs were isolated at 5 min (A,C) or 30 min (B,D) after fluid percussion injury or sham injury ($n = 6$ for each group). Diameters are expressed as absolute diameters (C,D) and as a percentage of the diameter at 100 mm Hg (A,B). *$p < 0.05$ compared to corresponding pressure in MCAs isolated from sham-injured rats using two-way repeated measures analysis of variance followed by Student-Newman-Keuls method for multiple comparisons of individual points.

at all pressure levels (Fig. 2). Repeated measures analysis of variance showed a significant treatment effect for MRI at 5 ($p = 0.0015$) and 30 min ($p = 0.0026$; Table 4) after TBI.

Inner diameters measured in Ca^{2+}-free PSS decreased sequentially as intraluminal pressure was reduced in all groups (Fig. 3). All four myogenic response curves were similar in Ca^{2+}-free PSS (Fig. 3).

DISCUSSION

These studies demonstrated normal vasodilatory responses to progressive reductions in intraluminal pressure *in vitro* in MCA segments harvested from sham-injured rats. In contrast, MCA segments harvested from rats 5 or 30 min after TBI exhibited vasoconstriction during pressure reductions. In fact, MCA segments from rats

TABLE 3. STATISTICAL RESULTS OF REPEATED MEASURES ANALYSIS OF VARIANCE OF THE PERCENT CHANGE IN DIAMETER OF MIDDLE CEREBRAL ARTERIES ISOLATED AT 5 MIN AND 30 MIN FOLLOWING SHAM INJURY AND FLUID PERCUSSION INJURY

Time	*Source*	*Degrees of freedom*	F *ratio*	p *value*
5 min	Treatment	1	15.07	*0.0031*
	Pressure	3	4.06	*0.0155*
	Interaction	3	2.97	*0.0477*
30 min	Treatment	1	23.41	*0.0007*
	Pressure	3	6.18	*0.0021*
	Interaction	3	5.63	*0.0035*

The diameters at different pressures (20, 40, 60, and 80 mm Hg) were expressed as a percent of the diameter when pressurized to 100 mm Hg. Significant *p* values are italicized.

subjected to moderate TBI decreased in diameter more during intraluminal pressure reductions in normal Ca^{2+}-containing than in Ca^{2+}-free PSS. This is the first demonstration of impaired vasodilatory responses *in vitro* in rodents after fluid percussion TBI.

Meininger and Davis (1992) defined spontaneous or intrinsic tone as a maintained state of partial smooth muscle activation and defined the myogenic response separately as a contraction of a blood vessel *in vitro* in response to increases in transmural pressure. Spontaneous tone developed in the MCAs in the present study as the arteries were warmed with 28°C to 37°C. The MCAs from both groups of injured rats had significantly ($p = 0.011$) larger diameters than the MCAs from both groups of sham-TBI rats. In contrast, MCA diameters measured in Ca^{2+}-free PSS were similar in all four groups (Fig. 3), suggesting that the MCAs were all of similar diameter in the absence of spontaneous tone but that the injured arteries developed less spontaneous tone.

The sham-injured groups exhibited sequential dilations as transmural pressure was reduced from 100 to 40 mm Hg (Fig. 1). When transmural pressure was further reduced from 40 to 20 mm Hg, the MCA diameters in both groups decreased but remained at or slightly above the resting (100 mm Hg) diameters. Spontaneous tone and myogenic dilations in both sham-TBI groups were abolished in the absence of Ca^{2+} (i.e., in Ca^{2+}-free PSS; Table 2, Fig. 3). Our observations are consistent with those of McCarron et al. (1989), who described Ca^{2+}-dependent myogenic responses to reductions in intraluminal pressure in rodent posterior cerebral arteries *in vitro*. Cerebral arterial and arterial branches of the MCA in the rodent (Harper et al., 1984) and feline (Kontos et al., 1978) pial circulation *in vivo* exhibit vasodilatory re-

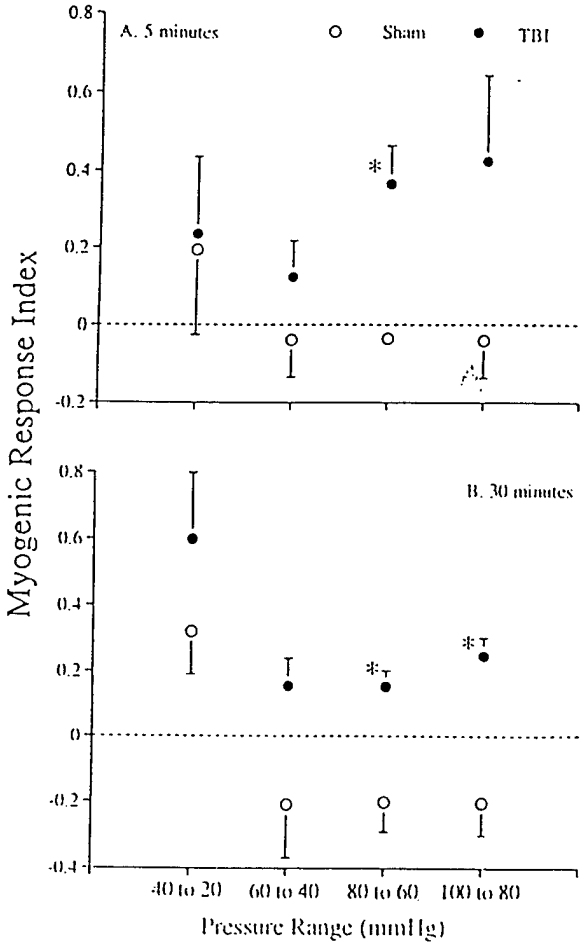

FIG. 2. Myogenic response index (MRI) is isolated middle cerebral arteries (MCAs). MCAs harvested 5 min (A) or 30 min (B) after fluid percussion injury or sham injury. $*p < 0.05$ compared to sham MCAs over the same pressure range using two-way repeated measures analysis of variance followed by Student-Newman-Keuls method for multiple comparisons of individual points.

TABLE 4. STATISTICAL RESULTS OF REPEATED MEASURES ANALYSIS OF VARIANCE OF THE MYOGENIC RESPONSE INDEX OF MIDDLE CEREBRAL ARTERIES ISOLATED AT 5 MIN AND 30 MIN FOLLOWING SHAM INJURY AND FLUID PERCUSSION INJURY

Time	*Source*	*Degrees of freedom*	F *ratio*	p *value*
5 min	Treatment	1	18.68	*0.0015*
	Pressure	3	0.449	0.7199
	Interaction	3	0.757	0.5272
30 min	Treatment	1	15.824	*0.0026*
	Pressure	3	8.307	*0.0004*
	Interaction	3	1.89	0.9031

Significant *p* values are italicized.

sponses to progressive hypotension. Therefore, the MCA segments harvested from sham-injured rats responded normally to reductions in intraluminal pressure.

The inner diameters of MCA segments harvested from injured rats were significantly ($p = 0.011$) larger than those taken from the sham-injured rats at intraluminal pressures of 100 mm Hg (Table 2, Fig. 3). The approximately 40% increases in resting diameters in the TBI groups are similar to vasodilation in the feline pial circulation observed after TBI (Wei et al., 1981; DeWitt et al., 1986). Mean arterial pressure increased significantly but transiently within 30 sec of TBI in both traumas. TBI-induced acute hypertension, believed to contribute to cerebral arterial vasodilation in feline *in vivo* FPI (Wei et al., 1980), may have contributed, in part, to the vasodilation that we observed in MCA *in vitro*. Acute hypertension may contribute to impaired cerebral vascular responses as well, but fluid percussion TBI impairs autoregulation in cats in the absence of acute, transient increases in arterial blood pressure (DeWitt et al., 1992).

Traumatic brain injury significantly reduced myogenic responses to progressive reductions in intraluminal pressure (Figs. 1–3). These observations are consistent with previous observations that TBI reduced vasodilatory responses in the feline pial circulation to systemic artial hypotension (Wei et al., 1980) *in vivo*. TBI also reduces or abolishes CBF autoregulation (DeWitt et al., 1992; Lewelt et al., 1980) *in vivo*, which would be consistent with our observations of reduced vasodilatory responses in isolated, pressurized MCAs *in vitro*. It is interesting to note that MCA diameters after TBI were reduced at each level of intraluminal pressure below the diameters observed in Ca^{2+}-free PSS (Fig. 3).

Since TBI produces oxygen free radicals (Fabian et al., 1998; Kontos and Wei, 1986; Wei et al., 1981) as well as prostanoids (DeWitt et al., 1988; Ellis et al., 1981, 1989), opiates (Armstead and Kurth, 1994; McIntosh et al., 1987a,b) and excitatory neurotransmitters (Faden et al., 1989), we investigated the hypothesis that cerebral arteries that were exposed to these (or other) agents *in situ* for 30 would be more affected than vessels that were only exposed for 5 min. The current studies demonstrated there were no significant differences in vasodilatory responses (Fig. 1) or MRIs (Fig. 2) in the MCAs harvested from rats 5 or 30 min after TBI, suggesting that the traumatic vascular injury that led to lost myogenic responses to hypotension had occurred within 5 min. Our results of no statistically reliable differences between the 5-min TBI and 30-min TBI groups also indicated that there was no significant recovery of myogenic responses with 30 min of TBI.

We reported (Bukoski et al., 1997) that vasodilatory responses to acetylcholine (ACh) and vasoconstrictor responses to serotonin and potassium chloride in ring seg-

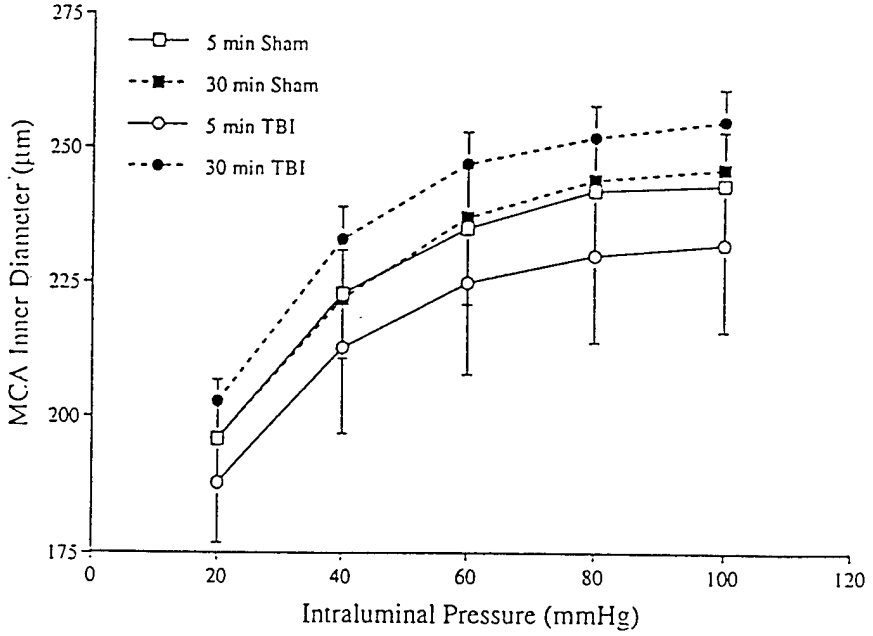

FIG. 3. Measurements of the absolute inner diameters of middle cerebral arteries (MCA) during progressive, sequential reductions in intraluminal pressure in Ca^{2+}-free PSS. Arteries were harvested from rats 5 or 30 min after sham injury or moderate, central, fluid percussion TBI ($n = 6$ rats per group). All diameters are expressed as mean ± SEM percent of baseline (100 mm Hg) diameter.

ments *in vitro* from posterior and middle cerebral arteries were not affected by either moderate (2.0 atm) or severe (3.0 atm) fluid percussion TBI. Previous reports indicate that TBI impairs cerebral vascular responses to ACh and serotonin in the feline pial circulation (Ellison et al., 1989; Kontos and Wei, 1992) *in vivo*. Therefore, while it appears that cerebral vascular smooth muscle is capable of constricting and dilating normally *in vitro* after TBI, trauma appears to affect pressurized arterial segments *in vitro* and intact cerebral arteries *in vivo* so as to reduce or abolish cerebral vascular responses to hypotension as well as to vasoactive receptor agonists such a serotonin and ACh. It should be noted that the present studies are not exactly comparable to previous studies because we did not test the effects of vasodilatory agents (i.e., ACh), while our earlier investigations did not assess myogenic responses after TBI. However, recent evidence from Golding et al. (1998) indicates that cortical impact injury enhances the sensitivity of vasodilatory purinoceptors *in vitro* without affecting vasodilatory responses to the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP). Thus, future studies should address the effects of fluid percussion TBI on vasodilatory receptors in isolated vessels. Additional future studies should investigate the effect of TBI on myogenic responses in arterial segments of several diameters since arterial segments of different diameters respond maximally at different levels of transmittal pressure in isolated preparations *in vitro* (Golding et al., 1998) as well as cranial window studies *in vivo* (Kontos et al., 1978).

CONCLUSION

Our results indicate that isolated, pressurized MCA segments exhibit normal vasodilatory responses to progressive reductions in transmural pressure *in vitro*. Further, our results indicate that fluid percussion TBI significantly reduces vasodilatory responses to reduced transmural pressure for at least 30 min after injury.

ACKNOWLEDGMENTS

We are grateful for the excellent technical assistance of Rachael Stephenson and Jennifer Chaisson and for the valuable secretarial support of Jordan Kicklighter. We thank Faith McLellan for editorial review. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. These studies were supported by grant number DAMD17-97-1-7008 from the Department of the Army and grant numbers NS19355 and NS27618 from the NIH.

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Peroxynitrite Reduces Vasodilatory Responses to Progressive Reductions in Intravascular Pressure in Isolated Middle Cerebral Arteries

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Acknowledgments

The authors are very grateful to Joseph S. Beckman, Ph.D., Department of Anesthesiology, University of Alabama, Birmingham, for providing the peroxynitrite used for these studies. The authors also thank Jordan Kicklighter for valuable editorial and secretarial support. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. These studies were supported by grant number DAMD17-97-1-7008 from the Department of the Army and grant numbers NS19355 and NS27618 from the NIH.

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Cover Title: $ONOO^-$ Impairs Cerebral Vasodilation

Number of Tables: 3

Number of Figures: 3

Type of Figures: Line Drawings (graphs)

Keywords: Cerebral arteries, free radicals, nitric oxide, oxidants, peroxynitrite

Abstract

Background and Purpose— In order to determine whether peroxynitrite ($ONOO^-$) impairs cerebral vasodilatory responses, the effects of different concentrations of $ONOO^-$ on myogenic vasodilatory responses to progressive reductions in intravascular pressure were determined in isolated rodent MCA's.

Methods—To test the effects of $ONOO^-$ on vasodilatory responses to progressive reductions in intravascular pressure, middle cerebral arteries (MCA's) from male Sprague-Dawley rats (n=30) were mounted in an arteriograph, pressurized to 50 mmHg and allowed to equilibrate for 1 h at 37°C. Intravascular pressure was increased to 100 mmHg and MCA inner diameters were measured as intravascular pressure was reduced sequentially from 100 mmHg to 20 mmHg in 20 mmHg increments. Intravascular pressure was returned to 100 mmHg and inactive or 10, 20 or 40 μM $ONOO^-$ was added to the bath and the sequence was repeated.

Results—$ONOO^-$, at all concentrations, caused a transient constriction of MCA's, followed by a marked vasodilation in the group exposed to 40μM $ONOO^-$. Inactive and 10 μM $ONOO^-$ did not affect vasodilatory responses to reduced intravascular pressure. MCA's treated with 20 and 40 μM $ONOO^-$ constricted in a pressure-dependent manner when intravascular pressure was reduced. $ONOO^-$, in the concentrations used in this study, had no effect on vasoconstriction to serotonin or vasodilation to potassium chloride.

Conclusions—These studies are the first to demonstrate that $ONOO^-$ reduces vasodilatory responses to progressive reductions in intravascular pressure in isolated MCA's in a concentration-dependent manner without reducing vasodilatory or vasoconstrictory responses to potassium chloride or serotonin, respectively.

The peroxynitrite anion ($ONOO^-$) is a powerful oxidizing agent [1,2] that, when protonated, decays to form a highly reactive hydroxyl-type radical. Peroxynitrite in its anionic form is remarkably stable, a factor that contributes to its toxicity by allowing it to diffuse farther from its site of formation in order to attack more distant cellular targets.[3] The hydroxyl radical, in contrast, can diffuse only a fraction (about 3 Å) of the typical diameter of a protein molecule,[4] thus making $ONOO^-$ the potentially more dangerous oxidant of the two. Peroxynitrite is formed when nitric oxide (NO) reacts with the superoxide anion radical ($\cdot O_2^-$)[5]. Superoxide is ubiquitous in the body and brain, and as much as 5% of total oxygen consumption has been estimated to produce $\cdot O_2^-$.[2,6] Superoxide is scavenged by superoxide dismutase (SOD) and, normally, the higher concentrations of SOD and $\cdot O_2^-$ favor the reaction between them. However, if NO concentrations increase, such as after cerebral ischemia,[7] the faster rate of the reaction between NO and $\cdot O_2^-$ will drive the formation of $ONOO^-$. Details of the chemistry of NO, $ONOO^-$ and $\cdot O_2^-$ are available in several excellent review articles.[1,2,4,8-10]

Peroxynitrite is capable of hydroxylation, nitration (+N), and nitrosation (a.k.a. nitrosylation, +NO) reactions.[1] Phenols react very readily with $ONOO^-$, and the most common phenol in biological tissues is tyrosine, which forms long-lived nitrotyrosine combinations when exposed to $ONOO^-$.[1,11,12] Peroxynitrite has been implicated in ischemia/reperfusion injury in both the rat lung [13] and brain.[3,14] L-NAME decreases lipid peroxidation in gerbils following cerebral ischemia, suggesting an involvement of NO and $ONOO^-$. DNA fragmentation by $ONOO^-$ results in the activation of poly-ADP-ribosyl transferase leading, ultimately, to cellular energy depletion in smooth muscle cells [15] and neurons.[16] $ONOO^-$ can react with cellular thiols,[17,18] thereby forming potential long-term NO donors that could interfere with NO-mediated cell signaling.[19]

At high concentrations, $ONOO^-$ damages rat coronary arterial endothelium, resulting in vasoconstriction; however, at lower concentrations, $ONOO^-$ causes coronary vasodilation.[20] Peroxynitrite activates guanylate cyclase in canine coronary arteries, and although the mechanism through which this activation takes place is not known, it may be due to the release of NO from tissues that have been nitrated by $ONOO^-$.[21] Topically applied $ONOO^-$ produced dose-dependent dilation (1-5 μM) in both large and small pial arterioles in cats.[22] In isolated rodent middle cerebral arteries (MCA's), $ONOO^-$ produced dose-dependent constriction, except at high concentrations, which caused vasodilation and impaired vasoconstrictor responses to serotonin.[23] The effects of $ONOO^-$ on physiological responses to changes in arterial pressure or carbon dioxide or on myogenic responses to changes in intravascular pressure in isolated vessels are unknown. In order to determine whether $ONOO^-$ impairs cerebral vasodilatory responses, the effects of different concentrations of $ONOO^-$ on myogenic vasodilatory responses to progressive reductions in intravascular pressure were determined in isolated rodent MCA's.

Subjects and Methods

Adult, male, Sprague-Dawley rats (300-400 g) were anesthetized with 4% isoflurane and decapitated. Brains were removed, and MCA's were harvested. Middle cerebral arteries were mounted in an arteriograph as previously described.[24] Briefly, a section (2 mm) of the artery was mounted in the arteriograph by inserting micropipettes into the lumen at either end and securing the vessel with nylon suture (10-0). All side branches were tied off. The mounted arterial segments were bathed in a physiological salt solution (PSS) of the following composition (NaCl = 130 mM; KCl = 4.7 mM; $MgSO_4 \cdot 7H_2O$ = 1.17 mM; glucose = 5 mM; $CaCl_2$ = 1.50 mM, $NaHCO_3$ = 15 mM). When gassed with a mixture of 74% N_2, 21% O_2, and 5% CO_2, this solution has a pH of $\approx$7.4, a PO_2 of $\approx$150, and a PCO_2 of $\approx$35. After mounting the arterial segment, the PSS was warmed from room temperature to 37°C and the arterial segments were allowed to equilibrate for 60 min, with intravascular pressure set at 50 mmHg by raising reservoir bottles connected to the micropipettes. Leaks were detected by monitoring intravascular pressure with the stopcocks to the reservoirs closed. A decline in pressure indicated a leak , and vessels with leaks that could not be tied off were excluded from further study. After 1 h of stabilization at an intravascular pressure of 50 mmHg, each vessel was exercised to decrease mechanical hysteresis by changing the pressure between 20 and 40 mmHg three times, allowing 5 min between pressure changes. The intravascular pressure was then increased to 100 mm Hg. The vessels were magnified with an inverted microscope equipped with a video camera and a monitor. Arterial inner diameter was measured using a video scaler calibrated with an optical micrometer. Vasodilatory responses were tested by decreasing intravascular pressure in 20 mmHg increments with a 10-min equilibration period at each pressure level before diameter measurements were made. After the initial myogenic responses were evaluated, intravascular pressure was returned to

100 mm Hg and the MCA's were exposed to one of the $ONOO^-$ concentrations described below. Peroxynitrite was added to the PSS and MCA diameters were measured 30 sec and 1, 2, and 10 minutes afterwards. Peroxynitrite was added only once, and the concentrations listed below take into account the volume of the arteriograph bath. Fifteen minutes after the addition of $ONOO^-$, vasodilatory responses were again tested by decreasing intravascular pressure in 20 mmHg increments from 100 mm Hg. Following the second measurement of vasodilatory responses to progressive reductions in intravascular pressure, serotonin (10^{-6} M) and then KCl (15 mM) were added. The Ca^{2+}-containing PSS was then replaced with Ca^{2+}-free PSS and sequential responses to progressive reductions in intravascular pressure were assessed again.

Experimental design

Rats were assigned to one of the following five groups (n = 6 rats per group):

Time control - Two sequential measurements of MCA diameters during progressive reduction in intravascular pressure were performed to determine whether a first test of vasodilatory responses to reduced intravascular pressure impaired responses during a second test.

Inactive $ONOO^-$ - MCA's were exposed to 20 μM $ONOO^-$ that had been added to PSS and allowed to remain at room temperature for 2 h. This causes a decomposition of $ONOO^-$ that has been confirmed spectrophotometrically.[23]

10 μM $ONOO^-$ - MCA's were exposed to 10 μM $ONOO^-$ added to the PSS bathing solution.

20 μM $ONOO^-$ - MCA's were exposed to 20 μM $ONOO^-$ added to the PSS bathing solution.

40 μM $ONOO^-$ - MCA's were exposed to 40 μM $ONOO^-$ added to the PSS bathing solution.

To determine whether the immediate vasoconstrictor effects of the $ONOO^-$ solutions were due to pH, 1 M NaOH was added to the bath and MCA diameters were determined 30 sec and 1, 2 and 10 min later (n = 6). In order to determine the pH and PO_2 of the NaOH and inactive and 10, 20 and 40 μM $ONOO^-$ solutions during the first 10 min of the experiment, NaOH or $ONOO^-$ were added to PSS in the arteriograph bath and samples were drawn 30 sec and 2, 5 and 10 min later and pH and pO_2 were measured. This sequence was repeated 3 times (Table 1).

Statistical analysis

Data were normalized to a percent of baseline diameters and analyzed using analysis of variance for a two-factor experiment with repeated measures on time after $ONOO^-$ exposure (0.5, 1, 2, and 10 min) or intravascular pressure (100, 80, 60, 40, and 20 mmHg). The other factor was the four treatment groups, i.e., $ONOO^-$ concentrations (inactive and 10μM, 20 μM, and 40 μM $ONOO^-$). Post-hoc analyses were performed using the Bonferroni/Dunn procedure for multiple comparisons. All data in the text, tables, and figures are expressed as mean ± standard error of the mean.

Results

The pH of the PSS in the arteriograph bath increased transiently and then returned to baseline values within 5 min of addition of NaOH, inactive $ONOO^-$ or 10, 20 and 40 μM $ONOO^-$ (Table 1). There were significant time effects on pH ($p < 0.0001$) but there were no significant differences in pH among the groups. The PO_2 of the PSS in the arteriograph bath also increased after the addition of NaOH, inactive $ONOO^-$ or 10, 20 and 40 μM $ONOO^-$ (Table 1). PO_2 changed significantly with time ($p < 0.0001$) after addition of the test solutions and the time vs. group interactions were significant ($p < 0.0001$) as well. Post hoc testing revealed that PO_2 was significantly higher in the 40μM $ONOO^-$ than in the NaOH or inactive $ONOO^-$ groups.

There were significant time ($p < 0.0001$) and time vs. group ($p < 0.0001$) effects for time after $ONOO^-$ exposure and $ONOO^-$ concentration (Fig. 1). Middle cerebral arterial diameters in the 40 μM $ONOO^-$ group were significantly different from MCA diameters in the 10 μM ($p = 0.010$) and the 20 μM ($p = 0.0003$) $ONOO^-$ concentrations. Exposure to any of the $ONOO^-$ concentrations caused constriction followed by some level of vasodilation. By 10 min after $ONOO^-$ addition to the bath, vasoconstriction in MCA's exposed to 10 μM $ONOO^-$ had decreased from 18 ± 5% to 3 ± 4% below baseline MCA diameters (Fig. 1). 20 μM $ONOO^-$ caused a 29 ± 3% constriction at 1 min after the addition of $ONOO^-$, followed by a slight (8 ± 7%) dilation by 10 min after $ONOO^-$ addition. 40 μM $ONOO^-$ produced a 31 ± 5% constriction 1 min after addition, followed by a dilation of 27 ± 11% and 17 ± 6% at 2 and 10 min, respectively. Exposure to inactive $ONOO^-$ and to NaOH produced transient reductions in diameter (21 ± 4% and 40 ± 8%, respectively), followed by a return towards baseline diameters.

In the time-control group, MCA diameters increased as intravascular pressure was reduced from 100 to 60 mmHg, decreased slightly as intravascular pressure was reduced from 60

to 40 and then to 20 mmHg (Fig. 2A). In both the first and the second time control measurements, MCA diameters were greater than baseline (i.e. MCAs dilated) even at intravascular pressures of 20 mmHg. There were no significant differences between the first and the second time control measurements. Exposure to inactive $ONOO^-$, which was maintained at room temperature for 2 h after addition to PSS before adding to the vessel bath, produced no significant ($p = 0.405$) change in the vasodilation produced by progressive reductions in intravascular pressure (Fig. 2B).

In all cases at baseline (before $ONOO^-$ exposure), MCA's dilated as intravascular pressure was reduced from 100 to 40 mmHg (Fig. 3, A-C, pretreatment). There was a significant treatment group vs. pressure interaction in the MCA's exposed to $ONOO^-$ ($p < 0.0001$). Exposure to 10 μM $ONOO^-$ did not significantly ($p = 0.617$) reduce vasodilatory responses to progressive reductions in intravascular pressure when compared to inactive $ONOO^-$ (Fig. 3A). In contrast, vasodilatory responses were significantly reduced by exposure to 20 μM (Fig. 3B, $p < 0.0001$) and 40 μM (3C, $p < 0.0001$) $ONOO^-$ when compared to inactive $ONOO^-$.

After the second measurements of MCA diameters during progressive reductions in intravascular pressure, pressure was returned to 100 mm Hg and baseline diameters were measured again. Serotonin (10^{-6} M) caused significant ($p < 0.0001$) reductions, compared to baseline, in the diameters (33 ± 6%, 39 ± 4%, 37 ± 4%, 34 ± 7%) of the MCA's exposed to inactive $ONOO^-$ and 10μM, 20 μM, and 40 μM $ONOO^-$, respectively (Table 2). Potassium chloride (15 mM) caused significant ($p < 0.0001$) vasodilation (196 ± 28%, 242 ± 18%, 188 ± 16%, 205 ± 35%) in preconstricted (with serotonin) MCA's exposed to inactive $ONOO^-$ and to 10 μM, 20 μM, and 40 μM $ONOO^-$, respectively (Table 2).

Replacement of the bathing solution with Ca^{2+}-free PSS caused marked vasodilation in all 4 groups (Table 3). Reductions in intravascular pressure caused progressive reductions in MCA diameters in all 4 groups. There were significant ($p < 0.0001$) pressure effects and a significant pressure vs. treatment group interaction ($p = 0.0144$). The group exposed to 10 μM $ONOO^-$ was significantly different ($p = 0.001$) from the group exposed to inactive $ONOO^-$. The other groups were not significantly different from one another.

Discussion

These results indicate that $ONOO^-$ impairs normal vasodilatory responses to reduced intravascular pressure in isolated rodent MCA's. In addition, we observed that impairment of the vasodilatory responses is concentration-dependent. 10 μM $ONOO^-$ produced no significant reductions in vasodilatory responses when compared to inactivated $ONOO^-$, while 20 μM and 40 μM $ONOO^-$ caused significant ($p < 0.0001$) reductions in MCA diameters during progressive reductions in intravascular pressure. 20 μM and 40 μM $ONOO^-$ impaired vasodilatory responses to reduced intravascular pressure without influencing the vasoconstrictory or vasodilatory responses to serotonin or KCl, respectively. These results represent the first report of the effects of $ONOO^-$ on cerebral vasodilatory responses, *in vitro*.

Concentrations of 10 μM and 20 μM $ONOO^-$ produced modest vasoconstriction (18 ± 5% and 29 ± 3%, respectively) within 0.5 min of addition to the water bath, followed by a return to (or nearly to) baseline diameters by 10 min after addition (Fig. 1). Elliott et al. [23] reported that 10 μM $ONOO^-$ produced a 19.5% constriction in MCA's harvested from Wistar rats anesthetized with sodium pentobarbital, which is very similar to the vasoconstriction we observed. Higher concentrations of $ONOO^-$ produced marked vasodilation.[23] We observed a 27% dilation 2 min after the addition of 40 μM $ONOO^-$ while Elliott et al. [23] reported a 43% dilation by 4 min after the addition of 50 μM $ONOO^-$. Wei et al. [22] reported vasodilation of small and large pial arterioles following the application of low concentrations (1-5 μM) of $ONOO^-$ in cats. These observations indicate that low concentrations of $ONOO^-$ produce vasoconstriction of isolated MCA's, *in vitro*, but produce vasodilation of pial arterioles, *in vivo*, while higher concentrations produce vasodilation of isolated MCA's, *in vitro*. Interestingly, high concentrations of $ONOO^-$ (100-150 μM) also cause pronounced vasodilation of pial arterioles, *in vivo*.[22] Therefore, $ONOO^-$,

in concentrations from 1 to 150 μM, causes cerebral arteriolar vasodilation, *in vivo*, while low concentrations (10-20 μM) produce cerebral arterial vasoconstriction and higher concentrations (>40 μM) cause vasodilation, *in vitro*.

The transient vasoconstriction observed after exposure to $ONOO^-$ may have been due to the effects of the pH of the test solutions rather than direct effects of $ONOO^-$. The pH of the PSS in the arteriograph bath increased significantly ($p < 0.0001$) but transiently after the addition of $ONOO^-$ or NaOH but then returned to baseline levels within 10 min (Table 1). Inactive $ONOO^-$ and PSS treated with NaOH produced vasoconstriction 0.5, 1 and 2 min after addition to the same degree as did $ONOO^-$. Ten minutes after exposure to inactive $ONOO^-$ or NaOH, MCA diameters returned towards baseline. These data suggest that the transient vasoconstriction caused by $ONOO^-$ was due to the alkalinity of the solutions rather than to a direct effect of $ONOO^-$. Vasoconstriction of cerebral arteries exposed to alkaline solutions has been reported in feline pial arterioles, *in vivo*,[25] and reductions in PCO_2 in the bathing medium cause membrane depolarization and vasoconstriction, *in vitro*.[26]

The most novel observation of this study is that $ONOO^-$ produces a concentration-dependent impairment of vasodilatory responses to reduced intravascular pressure in isolated, pressurized segments of the rodent MCA. The cerebral vasculature, as well as other vascular beds, maintains a resting state of contraction (tone) and is capable of intrinsic vasodilation or vasoconstriction in response to changes in intravascular pressure, *in vitro*. Meininger & Davis [27] defined spontaneous or intrinsic tone (i.e., myogenic tone) as a maintained state of partial smooth muscle activation and separately defined the myogenic response, *in vitro*, as a contraction of a blood vessel in response to increases in intravascular pressure. Direct myogenic responses to changes in intravascular pressure, *in vitro*, occur in the cerebral vasculature.[28-30] In the cerebral

circulation, myogenic tone and contraction in response to increases in intravascular pressure may involve voltage-dependent Ca^{2+} channels,[31] chloride channels [32] or the activation of phospholipase C.[33] Numerous mechanisms have been suggested to mediate myogenic tone and response to changes in intravascular pressure, but the mechanisms of these responses are currently unknown.

In most cases, during the initial (i.e. pretreatment) reductions in intravascular pressure, MCA's dilated as intravascular pressure was reduced to 40 mmHg and then constricted somewhat as intravascular pressure was reduced to 20 mmHg (Fig. 3, A-C). There were no significant differences in vasodilation between two successive measurements of MCA diameters during progressive reductions in intravascular pressure (Fig. 2A). MCA's exposed to inactive (Fig. 2B) or 10 μM $ONOO^-$ (Fig. 3A) dilated normally during progressive reductions in intravascular pressure. Vasodilation in response to reductions in intravascular pressure are consistent with the results of McCarron, et al. [30] who described Ca^{2+}-dependent vasodilatory responses to reductions in intravascular pressure in rodent posterior cerebral arteries, *in vitro*. Cerebral arterial and arteriolar branches of the MCA in the rodent [34] and feline [35] pial circulation, *in vivo*, exhibit vasodilatory responses to progressive hypotension. In the cerebral circulation, the degree of vasodilation is dependent on the pressure range and the size of the artery or arteriole studied, both *in vivo* [35] and *in vitro*.[36] The vasodilations of 5-10% of baseline that we observed pre-treatment or in the groups treated with inactive or 10 μM ONOO- are similiar to those reported for the same pressure ranges in MCA's in other *in vitro* studies in rats.[29,30,36] While 10 μM $ONOO^-$ didn't affect vasodilatory responses to reduced intravascular pressure (Fig. 3A), exposure to 20 and 40 μM led to vasoconstriction during progressive reductions in intravascular pressure (Fig. 3B & C). The vasoconstriction that we observed during reductions in intravascular pressure was not due to direct $ONOO^-$-induced vasoconstriction since 20 μM $ONOO^-$ didn't change MCA

diameter and 40 μM $ONOO^-$ produced a vasodilation (Fig. 1) at baseline (i.e., 100 mmHg) intravascular pressures. These observations indicate that $ONOO^-$ produces a concentration-dependent abolition of vasodilatory responses to decreases in intravascular pressure in MCA's.

Although $ONOO^-$ produced a concentration-dependent reduction in vasodilatory responses to progressive reductions in intravascular pressure, $ONOO^-$ exposure did not influence vasodilation or vasoconstriction in response to KCl or serotonin, respectively, added after washout of $ONOO^-$. Elliott et al. [23] observed that 5 to 10 μM $ONOO^-$, which produced vasoconstriction in MCA's, *in vitro*, did not affect vasoconstriction in response to 5 μM serotonin. In contrast, higher $ONOO^-$ concentrations (50-100 μM), which were associated with vasodilation of MCA's, progressively reduced vasoconstrictor responses to serotonin. Thus, low concentrations of $ONOO^-$ ($\leq$ 40 μM) produce selective impairment of vasodilatory responses to reduced intravascular pressure while not affecting other vasodilatory and vasoconstrictory responses in cerebral arteries. Higher $ONOO^-$ concentrations (> 50 μM) may cause injury that impairs multiple vasodilatory and vasoconstrictory responses. This hypothesis remains speculative, however, since we did not test the effects of high $ONOO^-$ concentrations and Elliott et al. [23] did not measure myogenic responses to changes in intravascular pressure.

Wei et al. [22] reported that the vasodilatory effects of $ONOO^-$ could be inhibited with glyburide, a blocker of ATP-sensitive potassium channels (K_{ATP}), and suggested that $ONOO^-$ may vasodilate by opening K_{ATP} channels. ATP-sensitive K^+ channels, which are present in the cerebral circulation, do not appear to contribute to resting tone [37] but may play some role in hypercapnic cerebral vasodilation.[38] Recent, preliminary evidence indicating that $ONOO^-$ inhibits K^+ currents in isolated, cerebral vascular smooth muscle cells suggests that $ONOO^-$ could interfere with K^+-mediated hyperpolarization and vasodilation.[39] Inhibitors of voltage-sensitive K^+

channels increased arterial constriction during increases in intravascular pressure.[40] $ONOO^-$ may act on K^+ channels contributing to the reductions in arterial diameter that we observed during progressive reductions in intravascular pressure. It is important to note, however, that because Knot and Nelson [40] studied increases while we studied decreases in intravascular pressure, their observations may not be applicable to the present study.

Insults to the cerebral vasculature such as experimental traumatic brain injury (TBI), *in vivo*, abolish vasodilatory responses to progressive systemic arterial hypotension in pial arteries and arterioles,[41] and TBI reduces cerebral blood flow autoregulation.[42,43] TBI also reduces myogenic vasodilatory responses to progressive reductions in intravascular pressure in middle cerebral arteries, *in vitro.*[44] Free radicals have been implicated in the effects of TBI on cerebral vascular reactivity.[45,46] Superoxide anion radicals are produced by TBI,[47] and TBI increases the activity of NO synthase [48] and the appearance of NO metabolites in brain microdialysate.[49] Therefore, precursors of $ONOO^-$ are produced by TBI and TBI and $ONOO^-$ produce similar reductions in myogenic vasodilatory responses to progressive reductions in intravascular pressure in isolated MCA's, suggesting that TBI-induced impairment of cerebral vascular reactivity, *in vivo*, may be partially attributable to $ONOO^-$.

Whether $ONOO^-$ levels, *in vivo*, reach the concentrations used in these *in vitro* studies remains to be determined. The half-life of $ONOO^-$ solutions with a pH of 7.4 at 37° C is about 1 sec (3). Therefore, the $ONOO^-$ concentration in contact with the MCA segments may have been less than the concentrations calculated based on the amount of stock solution added. Elliot et al. [23] estimated that the addition of 100 μM $ONOO^-$ resulted in an effective concentration of 15 μM $ONOO^-$ in the extracellular buffer of cell cultures. Interstitial concentrations of NO metabolites more than double after TBI in rats [49] and $\cdot O_2^-$ levels increase more than 5-fold.[50] As noted above,

the rapidity of the scavenging of $\cdot O_2^-$ by SOD prevents the formation of $ONOO^-$ until NO reaches micromolar concentrations,[2] suggesting that large increases in NOS activity would be required before $ONOO^-$ would be formed. Evidence of nitrotyrosine immunoreactivity in the rat myocardium after ischemia and reperfusion indicates that a doubling of NOS activity was associated with $ONOO^-$ formation.[51] While there appears to be sufficient NOS activity and $\cdot O_2^-$ formation to support the formation of $ONOO^-$ after TBI, $ONOO^-$ levels to which the cerebral vasculature is exposed to after TBI or cerebral ischemia are not known.

Conclusions

In summary, we have demonstrated that $ONOO^-$ exposure produces concentration-dependent reductions in vasodilatory responses to progressive reductions in intravascular pressure in isolated MCA's. Exposure to $ONOO^-$ also caused transient vasoconstriction that may have been caused by the alkaline pH of the vehicle solution for $ONOO^-$. These observations suggest that $ONOO^-$ may be the causative agent in trauma-induced impairment of cerebral vascular dilatory responses to systemic arterial hypotension (i.e. pressure autoregulation).

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TABLE 1. pH and PO_2 of the PSS in the arteriograph bath after addition of 10 μm, 20μM, 40μM, or inactive $ONOO^-$ or 15mM NaOH

		Time after addition (min)			
	Baseline	0.5	2	5	10
PH					
NaOH	7.44 ± 0.03	7.79 ± 0.01	7.79 ± 0.01	7.44 ± 0.02	7.43 ± 0.01
Inactive $OONO^-$	7.45 ± 0.01	7.77 ± 0.03	7.78 ± 0.02	7.45 ± 0.01	7.46 ± 0.01
10μM $OONO^-$	7.46 ± 0.01	7.68 ± 0.05	7.74 ± 0.04	7.50 ± 0.03	7.48 ± 0.01
20μM $OONO^-$	7.46 ± 0.01	7.77 ± 0.01	7.84 ± 0.01	7.48 ± 0.01	7.48 ± 0.01
40μM $OONO^-$	7.47 ± 0.01	8.06 ± 0.03	8.10 ± 0.02	7.48 ± 0.01	7.48 ± 0.01
PO_2 (mmHg)					
NaOH	159 ± 1*	174 ± 3*	173 ± 1*	155 ± 6*	158 ± 1*
Inactive $OONO^-$	156 ± 1†	193 ± 1†	179 ± 1†	153 ± 5†	152 ± 3†
10μM $OONO^-$	163 ± 1	180 ± 5	179 ± 5	173 ± 1	167 ± 1
20μM $OONO^-$	163 ± 2	194 ± 1	189 ± 1	173 ± 2	172 ± 1
40μM $OONO^-$	165 ± 1*†	216 ± 1*†	204 ± 3*†	174 ± 1*†	171 ± 1*†

*NaOH group was significantly different from the 40μM $OONO^-$ group.

†Inactive group was significantly different from the 40μM $OONO^-$ group.

TABLE 2. Responses to serotonin (5-HT, 10^{-6}M) or potassium chloride (KCl, 15 mM) in middle cerebral arteries (inner diameters, μm) exposed to 10μM, 20 μM, 40 μM, or inactive $ONOO^-$

$ONOO^-$	Baseline	5-HT	KCl
Inactive	102 ± 8	63 ± 3	123 ± 19
10 μM	122 ± 10	72 ± 3	174 ± 16
20 μM	108 ± 7	67 ± 2	125 ± 10
40 μM	128 ± 10	87 ± 16	168 ± 24

TABLE 3. Arterial inner diameters (μm) during progressive reductions in intravascular pressure in middle cerebral arteries in Ca^{2+}-free PSS after exposure to 10 μM, 20 μM, 40 μM, or inactive $ONOO^-$

$ONOO^-$	Intravascular Pressure (mmHg)				
	100	80	60	40	20
Inactive	259 ± 4	258 ± 4	253 ± 3	238 ± 3	200 ± 6
10 μM	279 ± 4*	278 ± 5*	272 ± 4*	258 ± 5*	219 ± 4*
20 μM	263 ± 3	262 ± 3	256 ± 3	242 ± 4	211 ± 3
40 μM	261 ± 3	260 ± 3	257 ± 4	245 ± 3	214 ± 4

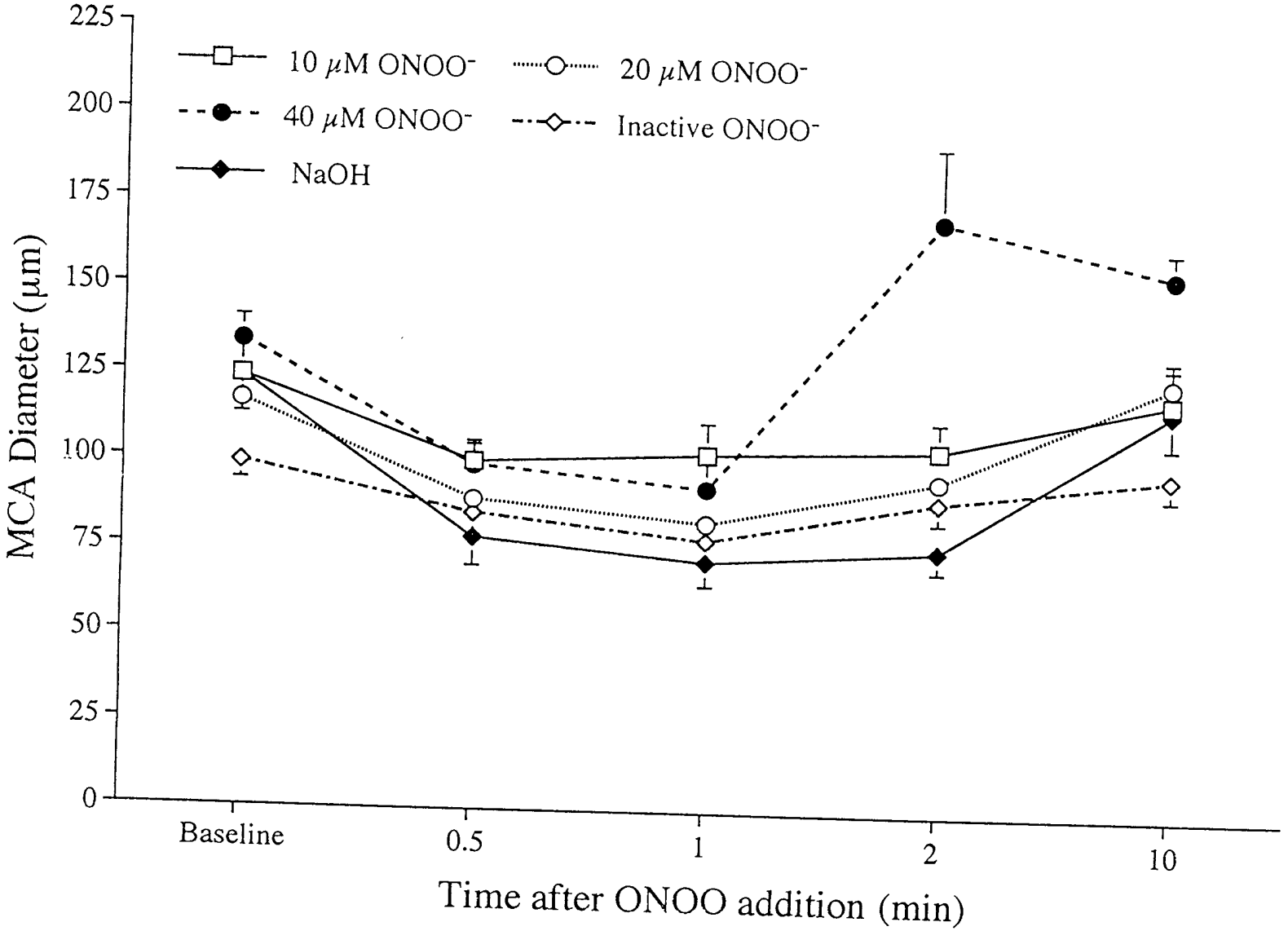
*$p < 0.05$ compared to inactive $OONO^-$ groups.

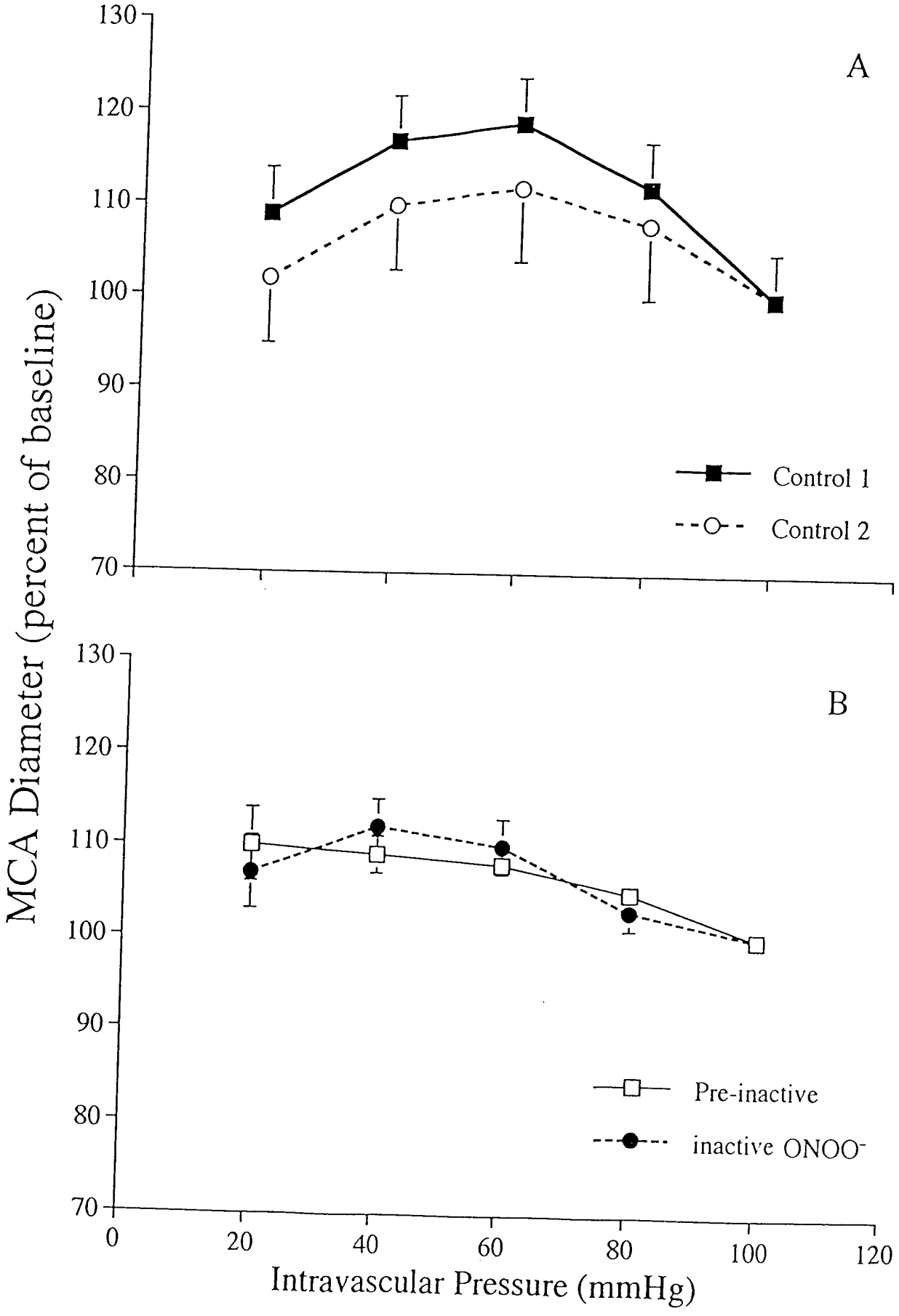
Figure Legends

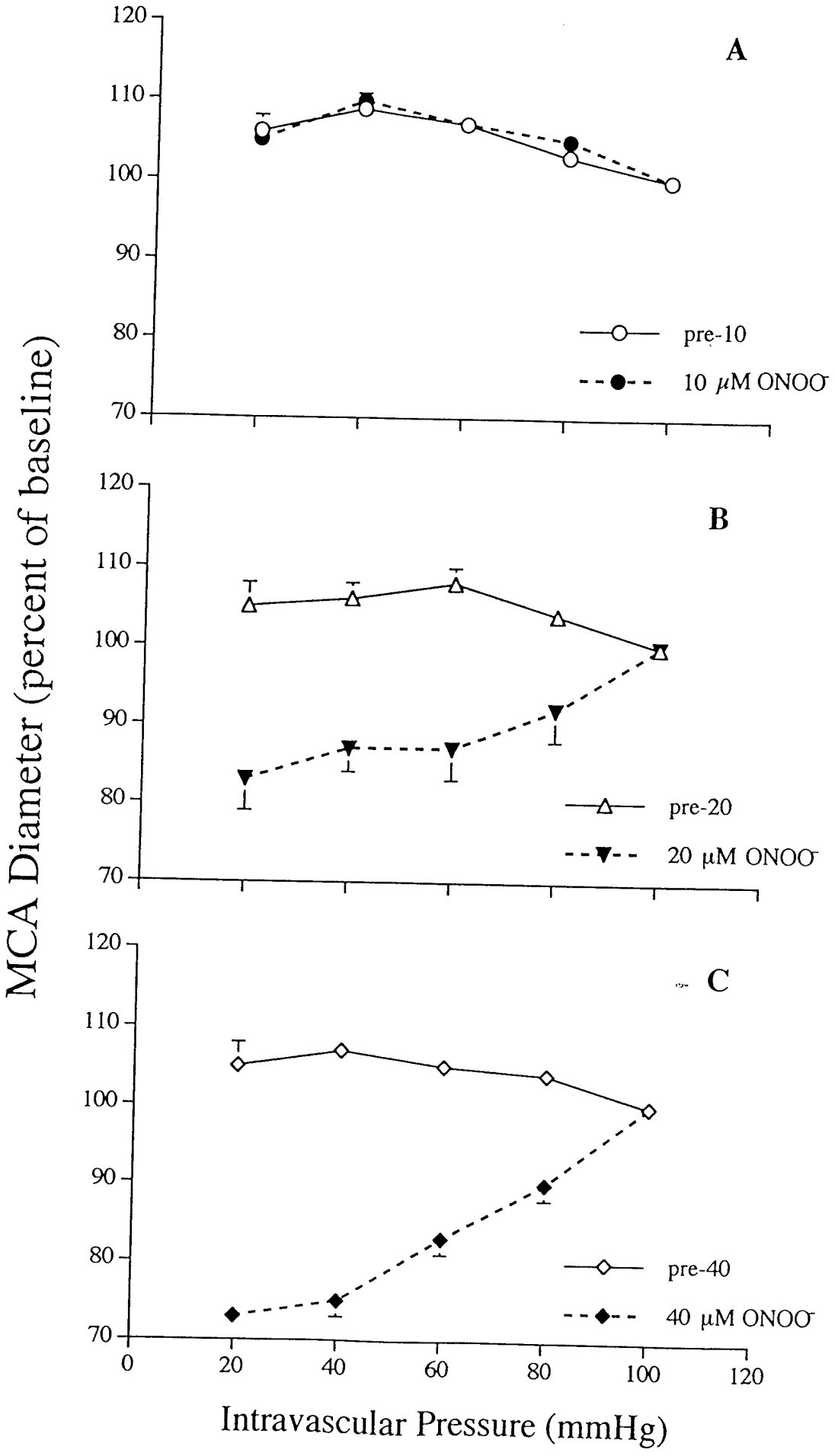
Figure 1. Middle cerebral artery (MCA) diameters 0.5, 1, 2 and 10 min after exposure to inactive peroxynitrite (inactive ONOO), to PSS with sodium hydroxide to raise the pH to that of the inactive $ONOO^-$ (NaOH) or to 10 μM, 20 μM or 40 μM $ONOO^-$.

Figure 2. Middle cerebral artery (MCA) diameters (percent of diameter at 100 mmHg) measured during two successive progressive reductions in intravascular pressure with no treatment (2A) or after exposure to inactive peroxynitrite (inactive $ONOO^-$, 2B).

Figure 3. Middle cerebral artery (MCA) diameters (percent of diameter at 100 mmHg) during progressive reductions in intravascular pressure after exposure to 10 μM (3A), 20 μM (3B), or 40 μM (3C) $ONOO^-$.


10 μM ONOO⁻
20 μM ONOO⁻
40 μM ONOO⁻
Inactive ONOO⁻
NaOH
MCA Diameter (μm)
225
200
175
150
125
100
75
50
25
0
Baseline
0.5
1
2
10
Time after ONOO addition (min)


A
B
MCA Diameter (percent of baseline)
Intravascular Pressure (mmHg)
130
120
110
100
90
80
70
0
20
40
60
80
100
120
Control 1
Control 2
Pre-inactive
inactive ONOO-


A
B
C
MCA Diameter (percent of baseline)
Intravascular Pressure (mmHg)
120
110
100
90
80
70
0
20
40
60
80
100
120
pre-10
10 μM ONOO⁻
pre-20
20 μM ONOO⁻
pre-40
40 μM ONOO⁻

Nitric Oxide Synthase Inhibition Does Not Inhibit Cerebral Vascular Responses to Hypotension or Hemodilution

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Running title: Nitric oxide, hypotension, and hemodilution

ABSTRACT

We investigated the effects of L-NMMA (N^G-monomethyl-L-arginine) and L-NAME (N^G-nitro-L-arginine methyl ester) on cerebral blood flow (CBF) and on CBF responses to hypotension and hemodilution. CBF was determined before and at 15, 30, 60 and 90 minutes intervals (microspheres) or continuously (laser Doppler) during an infusion of L-NAME (50 mg $\cdot kg^{-1} \cdot hr^{-1}$). To test autoregulation, rats were treated with saline, L-NMMA (25 mg/kg) or L-NAME (25 mg/kg) and CBF was measured before and after arterial pressure was lowered to 80 and 60 mmHg. To test for hyperemia to hemodilution, rats were treated with saline (n=6) or L-NAME (25 mg/kg, n=6) and CBF was measured before and after 30% and 60% of blood volume was exchanged for 0.9% saline. CBF decreased after 30 minutes of L-NAME infusion and remained constant for the next 60 minutes. CBF remained constant in all three groups as MAP was decreased. CBF increased 49% and 68% in the saline and L-NAME groups, respectively, following isovolemic replacement of 30 and 60% of total blood volume. Our data indicate that NOS inhibition decreases CBF but does not impair autoregulation or prevent hyperemia following hemodilution.

Key words: autoregulation, hemodilution, N^G-nitro-L-arginine methyl ester, nitric oxide, nitric oxide synthase inhibitors

INTRODUCTION

The endothelium-derived relaxing factor, nitric oxide (NO), may be involved in a variety of cerebral vasodilatory responses, including those generated by hypercapnia (1-5) and extracellular acidosis (6). Cerebral blood flow (CBF) increases secondary to increases in cerebral metabolic activity also may be mediated by NO (7,8), although there is some evidence to the contrary (9). The role of NO in cerebrovascular regulation has been extensively discussed elsewhere (10,11).

The role of NO in cerebral vasodilatory responses to arterial hypotension is uncertain. The vessels of the cerebral circulation dilate in response to systemic arterial hypotension, a response referred to as pressure autoregulation (12). Although the mechanisms contributing to autoregulation are not certain, myogenic, metabolic and intrinsic neural mechanisms have been suggested. The myogenic hypothesis states that cerebral vessels dilate or constrict owing to the responses of vascular smooth muscle cells to changes in transmural pressure (13,14). Intrinsic neural mechanisms suggest that perivascular nerves may contribute to or modulate autoregulatory responses (for review see reference (15)). The metabolic hypothesis states that products of local metabolic activity (i.e., H^+, CO_2, adenosine) may decrease cerebral vascular resistance in response to arterial hypotension (15), but none of the putative mediators have been demonstrated, unequivocally, to be involved. There is evidence that NO may mediate cerebral vasodilatory responses to hypotension (3,16). In addition to vasodilatory stimuli such as hypercapnia or hypoxia, decreases in hematocrit are associated with increases in CBF (17,18). The mechanism(s) of CBF increases during hemodilution are likely related to changes in blood oxygen content or to changes in extraction and blood viscosity, but the mediators are unknown

(19). We investigated whether inhibitors of NO synthase, an enzyme that produces NO from L-arginine, would reduce autoregulatory responses to controlled hemorrhagic hypotension or CBF increases to isovolemic hemodilution in rats.

METHODS

Surgical preparation for CBF measurement

In a protocol approved by the Animal Care and Use Committee of Wake Forest University Medical Center, male Sprague-Dawley rats (approximately 450 g) were anesthetized with methoxyflurane in an anesthetic chamber and their tracheas were intubated. Polyethylene cannulae were placed in both femoral arteries and veins for arterial reference sample withdrawal, arterial blood pressure recording, controlled hemorrhage, and drug infusion. The rats were paralyzed with pancuronium bromide (0.6 mg/kg i.v.) and mechanically ventilated with 1.5% to 2.0% isoflurane in oxygen and room air using a volume ventilator (EDCO Scientific, Inc., Chapel Hill, NC). Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Inc., Yellow Springs, OH) and maintained using a heating pad. A vinyl catheter (BOLAB, Inc., Lake Havasu City, AZ, od=0.039 in), was advanced into the left ventricle via the right axillary artery for injection of 15μm radiolabeled microspheres. At the completion of surgical preparation, isoflurane concentration was decreased to 1.0% to 1.5% in oxygen and air and the rats were allowed to stabilize for approximately 30 minutes during which time arterial pH, $PaCO_2$ and PaO_2 were monitored and maintained within normal limits using ventilatory rate and volume adjustments.

Cerebral blood flow was determined using 15μm polystyrene microspheres labeled with

^{113}Sn, ^{85}Sr, or ^{46}Sc (New England Nuclear, Dupont, Boston, MA) administered in random order. The vial containing the microspheres in 0.9% saline and 0.01% polyoxyethylene sorbitan monooleate (Tween 80) was agitated on a vortex mixer for 4 minutes. Three injections of approximately 10^5 microspheres/injection were performed in each rat at time intervals described below. Before and for 60 seconds after each injection, an arterial reference sample was withdrawn from the right femoral artery at a rate of 0.51 mL/min using a syringe pump (EDCO Scientific, Inc). At the conclusion of each experiment, rats were sacrificed using a barbiturate overdose. The brains were removed, separated into hemispheres and brain stems and their radioactivity was counted along with that of both kidneys and the arterial reference samples in a well-type gamma counter with a 3-in counting crystal (Autogamma 5000, Packard Instruments, Downers Grove, IL). Corrections for isotope overlap and calculations of corrected counts per minute (ccpm) were performed using a microcomputer connected to the gamma counter. Right and left renal blood flow (RBF) values were compared, and CBF determinations in which renal blood flows differed by more than 10% were discarded. Using the reference sample method, CBF and renal blood flow (RBF) were calculated according to the formula (20):

$$BF\ (mL \cdot min^{-1} \cdot 100\ g^{-1}) = \frac{Cb \cdot ARR \cdot 100}{Cr \cdot Wb}$$

where BF = tissue blood flow; Cb = counts in tissue sample; Cr = counts in reference arterial sample; ARR = arterial reference withdrawal rate; Wb = weight of tissue sample. Total CBF (tCBF) was calculated as a weighted average of regional CBF.

In a separate group of rats, CBF was monitored continuously using laser Doppler flowmetry (LDF). The anesthetized rats were surgically prepared for measurement of relative

perfusion, as described elsewhere (21). Briefly, the left calvaria lateral to the midsagittal suture was thinned using an air-cooled drill (Dremel, Racine, WI). Using an electrode holder on a stereotaxic headholder (Stoelting Co., Wood Dale, IL) a fiberoptic needle probe (Perimed, Stockholm, Sweden) was placed over the shaved parietal calvaria and carefully positioned away from large vessels visible in the remaining calvaria. The probe emits monochromatic red light (632.8 nm), which is reflected by moving erythrocytes. The power and frequency of the reflected signal, detected by optical sensors in the needle probe head, are proportional to the blood volume and blood velocity, respectively. Blood velocity is calculated based upon the Doppler shift created by red blood cells moving in the area perfused by the probe laser and reflected back to the receiver in the same probe. Perfusion is calculated as the product of blood volume and velocity in a 1 mm^3 tissue volume under the probe (22). Measurements were recorded on a PeriFlux PF3 Laser Doppler Perfusion Monitor (Perimed). Values for LDF were compared between rats based on a percentage change from baseline values after the experimental procedure.

Nitric oxide synthase inhibitors

To determine the contribution of NO to autoregulatory vasodilation rats were treated with stereoisomers of L-arginine, N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl (L-NAME), which competitively inhibit the synthesis of NO in the porcine aorta (23). For the subsequent tests of the effect of NOS inhibition on hyperemia in response to hemodilution, only L-NAME was used because L-NMMA may be converted to arginine and serve as a NOS substrate as well as a NOS inhibitor (24,25).

Experimental design

All rats were anesthetized and prepared for microsphere CBF determinations, as described above and the following experiments were performed:

In order to determine the dose of L-NAME that maximally reduces CBF, Group MS (microsphere CBF measured) rats received continuous intravenous infusions of L-NAME (50 $mg \cdot kg^{-1} \cdot hr^{-1}$). Because CBF could be determined only three times in each rat, two subgroups were created in which CBF was measured before and 15 and 30 minutes after the beginning of L-NAME infusion (n=8) or 30, 60 and 90 minutes after infusion (n=7).

Group LD (laser Doppler CBF measurement) rats (n=8) received continuous intravenous infusions of L-NAME (50 $mg \cdot kg^{-1} \cdot hr^{-1}$) and CBF was measured continuously using LDF. Relative perfusion was calculated as a percent of baseline (pre-L-NAME) perfusion averaged over a 1-minute period every 15 minutes for 90 minutes after the beginning of L-NAME infusion. These studies involve the continuous measurement of relative perfusion were performed to confirm the intermittent but quantitative microsphere studies (Group MS).

Group A (autoregulation) rats randomly received intravenous injections of either 0.9% saline (n=7), L-NMMA (25 mg/kg, n=7) or L-NAME (25 mg/kg, n=8), with CBF determined 15 minutes after treatment. Mean arterial pressure (MAP) was reduced by controlled hemorrhage to 80 and then 60 mm Hg and CBF was measured at each interval.

Group H (hemodilution) rats randomly received intravenous injections of either 0.9% saline (n=8) or L-NAME (25 mg/kg, n=9). Cerebral blood flood was determined 15 minutes afterward and then arterial hemoglobin (Hgb) concentration was reduced by isovolemic

hemodilution with 0.9% saline to 70% and then 40% of baseline Hgb. Cerebral blood flow was measured at baseline and at each level of hemodilution.

Statistical methods

Group MS

Data were analyzed in a manner analogous to a randomized complete-block design for each subgroup. Animal number was the blocking factor and time after infusion was the repeated measurement factor.

Group LD

Data were analyzed using a one-way analysis of variance, with time after infusion as the measurement factor.

Group A

Data were analyzed using an analysis of variance procedure for a two-factor experiment, with repeated measures on one factor (level of controlled hypotension). The two factors were defined as treatment (saline, L-NMMA and L-NAME) and MAP (level of hypotension = baseline, MAP 80 or MAP 60).

Group H

Data were analyzed using an analysis of variance procedure for a two-factor experiment, with repeated measures on one factor (Hgb concentration). The two factors were defined as

treatment (saline and L-NAME) and Hgb concentration (0, 30, or 60 percent of blood volume replaced).

Fisher's least-significant difference procedure with Bonferroni adjustments for number of comparisons were used for multiple comparison. Tests, effects and interactions were assessed at the 0.05 level of significance.

RESULTS

Group MS

Arterial CO_2, O_2, pH and Hgb concentrations remained constant over the course of the L-NAME infusion (Table 1). Mean arterial pressure increased significantly ($p < 0.05$) within 15 minutes of the onset of L-NAME infusion in both groups (Table 1). Total CBF (tCBF) decreased during L-NAME infusion (Figure 1), but CBF was not significantly different from baseline after 30 minutes of infusion ($p < 0.06$). tCBF decreased further after 60 minutes and then leveled off at 90 minutes of L-NAME infusion. Because the 60- and 90-minute time points were measured in different animals than the baseline measurements, they could not be statistically compared with each other. Regional CBF, in all brain regions studied, followed a pattern similar to that of tCBF (Table 2).

Group LD

Arterial CO_2, O_2, pH and Hgb concentrations remained constant over the course of L-NAME infusion (Table 1). Mean arterial pressure increased significantly within 15 after the beginning of the L-NAME infusion. Cerebral blood flow was significantly lower than baseline

after 30, 60 and 90 minutes of L-NAME infusion (Figure 1).

Group A

Arterial CO_2, O_2, pH and Hgb concentrations remained constant over the course of the CBF measurements in all three groups (Table 3). Baseline MAP was higher and tCBF was lower in the subgroups treated with NO synthase (NOS) inhibitors than in the saline-treated subgroup, but the differences were not statistically significant. Total CBF increased slightly in all three groups at a MAP of 80 mm Hg and then decreased slightly (saline-treated rats) or remained slightly higher than baseline (L-NMMA- and L-NAME-treated rats) at a MAP of 60 mm Hg (Figure 2). Brain stem and hemispheric blood flows were slightly lower and higher than tCBF, respectively, but both remained constant in all three subgroups as MAP was lowered to 80 and 60 mm Hg (Table 2).

Group H

Arterial CO_2, O_2 and pH and rectal temperature remained constant over the course of the CBF measurements in both groups (Table 4). Mean arterial pressure was significantly higher ($p < 0.05$) in the L-NAME treated rats at all three measurement intervals. Total CBF increased significantly ($p < 0.05$) in both saline-treated and L-NAME-treated rats as 30 and 60% of estimated fluid volume was replaced with 0.9% saline (Fig. 3). Although baseline tCBF was lower in the L-NAME treated rats, the tCBF increase in the L-NAME-treated group was greater at 60% hemodilution when expressed as percent of baseline tCBF (49% and 68% higher than baseline in saline and L-NAME groups, respectively). Brain stem and hemispheric CBF

increased in parallel with tCBF in both subgroups (Table 2).

DISCUSSION

The NOS inhibitor L-NAME, in concentrations that significantly and maximally reduced CBF, did not significantly alter cerebral vascular responses to controlled, sequential arterial hypotension. In addition, autoregulatory responses to arterial hypotension were not affected by L-NMMA, another nonspecific inhibitor of NOS. We also observed that L-NAME did not reduce hyperemia in response to hemodilution. This is the first report of the effects of NOS inhibition on moderate and severe levels of hemodilution in rats.

The results of these laser Doppler studies, which demonstrated that the administration of an inhibitor of NO synthase (L-NAME) decreases CBF in isoflurane-anesthetized rats, are consistent with a resting vasodilatory "tone" mediated by NO. In contrast, inhibition of NO synthesis (L-NMMA or L-NAME) did not prevent vasodilatory responses to progressive hemorrhagic hypotension (i.e., autoregulation). In addition, the CBF increases that occur after isovolemic hemodilution were not prevented by the NO synthase inhibitor L-NAME.

There is considerable evidence that the rodent cerebral circulation exhibits a resting vasodilatory tone mediated by NO (1,2,26-29). Beckman et al. (26) reported that CBF decreased markedly after the infusion of L-nitroarginine hydrochloride (30 mg/kg i.v.) in halothane-anesthetized rats. Tanaka et al. (27), who measured local CBF using iodoantipyrine in awake rats receiving L-NMMA (25 mg/kg i.v.), found that local CBF was decreased from 20% (substantia nigra) to 33% (hypothalamus) when compared with a saline-treated group. Faraci (28) reported that cerebral arteries and arterioles in barbiturate-anesthetized rats decreased in

diameter after topical application of L-NMMA. Kozniewska et al. (29) observed that CBF, measured using intracarotid ^{133}Xe in rats anesthetized with chloral hydrate, decreased approximately 21% after L-NMMA administration (100 mg/kg i.v.). They also reported that the effects of L-NMMA on CBF could be reversed using L-arginine (300 mg/kg i.v.) but not D-arginine (300 mg/kg i.v.). Wang et al. (1), using halothane-anesthetized rats, found that N^G-nitro-L-arginine decreased CBF (intracarotid ^{133}Xe) in a dose-dependent manner and that CBF decreases persisted for at least 2 hours. Pelligrino and colleagues (2) reported decreases in regional CBF (radioactive microspheres) of more than 50% after infusion of L-NAME (3 mg $\cdot$ $kg^{-1} \cdot min^{-1}$) in rats anesthetized with 70% N_2O and fentanyl (25 $\mu g \cdot kg^{-1} \cdot hr^{-1}$). Autoradiographic studies indicate that L-NAME (30 mg/kg) infusion decreases CBF by 17% (parietal cortex) to 49% (hypophysis) across different brain regions in rats (30). These studies, which were performed using a variety of anesthetic and CBF measurement techniques, consistently demonstrate that the inhibition of NO synthesis decreases CBF and support the hypothesis that there is a resting vasodilatory tone mediated by NO in the rodent cerebral circulation.

Although there is clear evidence of an NO-mediated vasodilatory tone in rodents, the data from investigations in other species are less clear. Nitro-arginine infusion in awake sheep had no effect on CBF (radioactive microspheres) but the doses of nitro-arginine (0.1 mg/kg) were much lower than those used in other studies (31). In cats anesthetized with chloralose and urethane (50 mg/kg and 200 mg/kg, respectively), nitro-arginine (30 mg/kg + 1 mg $\cdot$ $kg^{-1} \cdot min^{-1}$) decreased regional CBF (radioactive microspheres) significantly in the cerebellum, medulla oblongata and spinal cord but had no effect on cortical blood flow (32). L-NAME (20

mg/kg) caused no significant changes in regional CBF (radioactive microspheres) in dogs anesthetized with sodium pentobarbital (50 mg/kg) (33). It is unclear whether these negative results are due to interspecies variability or to differences in doses of the NO inhibitor (31) or in anesthetics (32,33).

Inhibition of NO synthase did not alter compensatory cerebral vasodilatory responses to progressive hypotension (Group A, Figure 2). These observations indicate that NO is not the primary mediator of autoregulation, at least in the range of arterial blood pressures studied here. The present studies are in agreement with the results of Wang et al. (16), who reported that inhibition of NO synthase with nitro-arginine had no effect on autoregulation in halothane-anesthetized rats. Buchanan and Phillis (3), using a venous outflow preparation in rats, observed no changes in responses to hypotension after treatment with L-NAME. Takahashi et al. (34) reported that chronic administration of L-NAME (twice daily for 4 days) does not reduce autoregulatory responses in conscious rats. The NOS inhibitors L-NMMA or nitro-L-arginine reduced vasodilatory responses to hypercapnia but did not affect autoregulation in primates (35). Our studies, performed using a different anesthetic, CBF measurement method and L-NAME as well as L-NMMA, confirm the results of these previous investigators. Careful examination of the CBF data from Wang et al. (see figure 1 in reference (16)) reveals that, although the autoregulatory curves were identical in nitro-arginine treated and untreated rats, there were a larger number of low (< 50% of baseline) CBF values at the lower levels of arterial blood pressure in the nitro-arginine treated rats. This observation suggests that, while NO is not involved in autoregulation to higher blood pressure levels, there may be an NO-dependent component of vasodilatory responses to very low levels of arterial blood pressure. Although

recent preliminary evidence suggests that the specific neuronal NOS inhibitor, 7-nitroindazole, does not affect the lower limit of autoregulation in rats (36), the suggestion that NO may be involved in part, though not all, of the autoregulatory response is supported by evidence that topical application of nitro-L-arginine reduces autoregulatory responses during severe hypotension in rats (37,38).

An analogous mechanism has been suggested by Pelligrino et al. (2) to explain why the hypoxic vasodilatory response seems to be NO-dependent or NO-independent depending on the levels of PaO_2 studied (see also reference (16)). This intriguing hypothesis related to the hypoxic vasodilatory response may not be applicable to autoregulatory vasodilatory responses. If vasodilatory responses to the lower levels of arterial pressure were NO-dependent, then the vessels that contribute most to CBF control at those pressure levels would be the most sensitive to inhibition of NO synthesis. In fact, the opposite is true, as the vasodilatory responses to lower levels of arterial blood pressure seem to reside in the smallest arteries and arterioles (39) which are the least affected by inhibition of NO synthesis (28).

There is additional evidence that inhibition of NOS results in significant decreases in local CBF during hemorrhagic hypotension (40), suggesting a role for NO in autoregulation. Tanaka et al. (40) measured CBF at one level of hypotension using ^{14}C-iodoantipyrine in awake rats treated with L-NMMA. Because they failed to measure CBF during hypotension in the absence of L-NMMA (they referred to autoradiographic results from a previous study using a different level of hypotension) it is difficult to interpret their observations. Perhaps treatment with NOS inhibitors shifts the autoregulatory curve but Wang et al. (16), who measured CBF repeatedly across a wide range of values, observed no shift in the lower limit of autoregulation.

It is possible that, unlike Tanaka et al. (40), we failed to inhibit NO production, but this seems unlikely as we used comparable doses of L-NMMA and observed CBF decreases similar to those they reported.

Our data indicate that inhibition of NOS does not prevent increases in CBF that occur after hemodilution. In fact, when expressed as a percent of baseline, the L-NAME-treated group exhibited a greater increase than did the saline-treated group (68% and 49% increases in L-NAME and saline groups, respectively, calculated from Table 2). It is unclear why the inhibition of NOS would increase CBF in response to hemodilution. Pelligrino et al. (2) observed similar enhancement of CBF increases to hypoxia in L-NAME-treated rats. Our results are consistent with those of Todd et al. (41) who reported that L-NAME, at doses that reduce NOS activity by about 65%, did not reduce hyperemia responses to hemodilution in rabbits.

Hemodilution is normally associated with compensatory increases in CBF (18). The difference between cerebral vascular responses to hypotension and hemodilution is that hypotension is associated with vasodilation (i.e., increases in pial arterial and arteriolar diameter) while CBF increases after hemodilution appear to be associated with cerebral vasoconstriction or with no change in cerebral vessel diameter (42-44). The mechanism by which hemodilution increases CBF is unknown but increases in blood flow in any vascular bed require either that vascular resistance decrease or that blood velocity increase. Cerebral blood flow increases during hemodilution are unlikely to be due to cerebral vasodilation as cerebral arteries and arterioles throughout the cerebral vascular bed either decrease or remain constant in diameter as hematocrit is reduced (42-44). Cerebral blood flow increases during hemodilution likely result from decreases in blood viscosity (42,44). Because decreases in hematocrit have been reported

to increase cerebral blood velocity (45), it seems probable that increased CBF during hemodilution results from increases in cerebral blood velocity secondary to decreases in blood viscosity. As inhibition of NO synthase would not affect blood viscosity and, therefore, blood velocity, it is not surprising that L-NAME did not prevent CBF increases to hemodilution.

It is possible that, while NO is not the primary vasodilator associated with cerebral vascular responses to hypotension, some basal levels of NO are required for normal autoregulation function. Bryan et al. (46) reported that endothelial removal or NOS inhibition blocked vasodilatory responses to α_2-adrenergic agonists in isolated, pressurized middle cerebral artery segments but that the addition of sub-vasodilatory concentrations of NO would restore normal vasodilation. They concluded that NO was playing a permissive rather than a primary role in α_2-adrenergic vasodilatory responses; similar permissive effects of NO have been suggested to mediate vasodilatory responses to hypercapnia (47) and volatile anesthetics (48). Recently, Wang et al. (49) provided compelling evidence that redundant vasodilatory mechanisms exist in the cerebral circulation and that NOS inhibition may unmask these underlying mechanisms. Although the dose of L-NAME used in our studies of vasodilatory responses to hypotension and hemodilution were sufficient to maximally reduce resting CBF (i.e., higher doses in Groups MS and LD did not produce further CBF reductions) they were likely insufficient to completely inhibit NOS. Iadecola et al. (50) reported that 40 mg/kg L-NAME reduced NOS activity only by 50% in rats. Therefore, the present studies cannot exclude the possibility that sufficient NOS activity remained after L-NMMA or L-NAME treatment to allow normal vasodilatory responses to hypotension and hemodilution.

In summary, our data indicate that inhibition of NOS decreases CBF but does not impair

autoregulation or prevent the compensatory increases in CBF that occur after isovolemic hemodilution. Our results do not exclude involvement of NO in autoregulatory responses to hypotension or in CBF increases to hemodilution, but they do suggest that NO is not the primary or sole mediator of these important cerebral vascular compensatory responses.

ACKNOWLEDGMENTS

This work was performed at the Basic Cerebrovascular Research Laboratories of the Department of Anesthesia, Wake Forest University School of Medicine, and was supported in part by NIH/NINDS grant 19355 and grant DAMD17-97-1-7008 from the Department of the Army.

We thank Faith McLellan for editorial assistance and Jordan Kicklighter for help with organizing and typing this manuscript. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories and to Francis M. James, III, M.D., and our colleagues in the Department of Anesthesia at Wake Forest University Medical Center for their support of the Basic Cerebrovascular Laboratories.

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TABLE 1. Arterial blood parameters in rats during a continuous infusion of L-NAME ($50\ mg \cdot kg^{-1} \cdot min^{-1}$).

Parameter	Group	Minutes of L-NAME infusion				
		Baseline	15	30	60	90
$PaCO_2$	MS	38.4 ± 1.3	36.2 ± 1.6	38.0 ± 0.8	36.5 ± 0.8	39.4 ± 1.0
(mm Hg)	LD	34.8 ± 1.0	—	—	—	40.7 ± 1.0*
PaO_2	MS	120 ± 8	127 ± 7	128 ± 5	132 ± 7	180 ± 6
(mm Hg)	LD	298 ± 9	—	—	—	280 ± 16
pH	MS	7.43 ± 0.01	7.41 ± 0.01	7.40 ± 0.01	7.42 ± 0.01	7.39 ±0.02
	LD	7.45 ± 0.3	—	—	—	7.38 ± 0.01
Hgb	MS	14.3 ± 0.4	—	—	—	13.8 ± 0.3
(gm/dL)	LD	14.2 ± 0.3	—	—	—	14.8 ± 0.4
MAP	MS	108 ± 3	130 ± 3*	140 ± 2*	143 ± 5	149 ± 3
(mm Hg)	LD	120 ± 4	145 ± 6*	156 ± 5*	163 ± 3*	167 ± 5*

All values are mean ± SEM. Group MS = Radioactive microsphere CBF measurement; Group LD = Laser Doppler CBF measurement. * = significantly ($p < 0.05$) different from baseline.

TABLE 2. Total, hemispheric (hemi) and brain stem (br st) blood flow ($mL \cdot min^{-1} \cdot 100g^{-1}$) during L-NAME infusion (Group MS), during controlled hypotension with NOS inhibition (Group A) and during blood replacement (Group H) with L-NAME treatment.

Group MS

Region	Time after L-NAME infusion (minutes)				
	0	15	30	60	90
Total CBF	120 ± 13	106 ± 10	93 ± 8	76 ± 9	86 ±11
Hemi CBF	131 ± 16	108 ± 10	91 ± 8	76 ± 10	83 ±11
Br st CBF	110 ± 12	102 ± 10	95 ± 8	72 ± 9	88 ±10

Group A

Treatment	Region	Hemorrhagic Hypotension		
		Baseline	80 mm Hg	60 mm Hg
Saline	Total CBF	130 ± 15	128 ± 17	109 ± 14
	Hemi CBF	135 ± 15	133 ± 18	118 ± 17
	Br st CBF	126 ± 15	125 ± 19	92 ± 12
L-NMMA	Total CBF	112 ± 17	130 ± 17	121 ± 9
	Hemi CBF	117 ± 18	133 ± 17	118 ± 10
	Br st CBF	107 ± 18	131 ± 22	133 ± 8

L-NAME	Total CBF	110 ± 10	112 ± 7	112 ± 9
	Hemi CBF	110 ± 11	115 ± 6	114 ± 9
	Br st CBF	114 ± 12	100 ± 9	111± 10

Group H

Treatment	Region	Blood volume replaced (%)		
		0	30	60
Saline	Total CBF	134 ± 11	147 ± 12	200 ± 14
	Hemi CBF	141 ± 12	152 ± 13	209 ± 16
	Br st CBF	128 ± 11	138 ± 12	181 ± 14
L-NAME	Total CBF	104 ± 11	106 ± 8	175 ± 15
	Hemi CBF	107 ± 13	108 ± 7	186 ± 18
	Br st CBF	99 ± 8	101 ± 5	151 ± 15

All values are mean ± SEM.

TABLE 3. Arterial blood parameters in rats (Group A) treated with L-NMMA (25 mg/kg) or L-NAME (25 mg/kg) during hemorrhage hypotension.

Parameter	Hemorrhagic hypotension			
	Subgroup	Baseline	MAP 80	MAP 60
$PaCO_2$ (mm Hg)	Saline	40.1 ± 1.7	36.8 ± 1.12	36.8 ± 1.0
	L-NMMA	38.8 ± 2.4	37.5 ± 1.4	38.5 ± 1.1
	L-NAME	42.2 ± 2.0	35.9 ± 2.6	36.3 ± 3.2
PaO_2 (mm Hg)	Saline	121 ± 11	124 ± 6	141 ± 7
	L-NMMA	119 ± 5	119 ± 3	131 ± 6
	L-NAME	124 ± 15	165 ± 13	182 ± 13
pH	Saline	7.40 ± 0.02	7.40 ± 0.01	7.40 ± 0.01
	L-NMMA	7.42 ± 0.02	7.40 ± 0.01	7.39 ± 0.01
	L-NAME	7.38 ± 0.02	7.38 ± 0.03	7.30 ± 0.04
Hgb (g/dL)	Saline	14.4 ± 0.4	—	12.2 ± 0.4
	L-NMMA	13.9 ± 0.3	—	11.6 ± 0.3
	L-NAME	13.4 ± 0.2	—	10.4 ± 0.2
MAP (mm Hg)	Saline	94 ± 14	70 ± 12	64 ± 4*
	L-NMMA	111 ± 10	73 ± 5	60 ± 3*
	L-NAME	133 ± 5	83 ± 2	62 ± 4*

All values are mean ± SEM. * significantly different ($p < 0.05$) from baseline.

TABLE 4. Arterial blood parameters in rats (Group H) treated with L-NAME (25 mg/kg) during isovolemic hemodilution.

Parameter		Percent baseline Hgb concentrations		
	Subgroup	100 (baseline)	70	40
$PaCO_2$	Saline	40.5 ± 2.0	37.7 ± 1.4	37.6 ± 1.6
(mm Hg)	L-NAME	40.3 ± 2.2	37.3 ± 0.8	37.6 ± 1.6
PaO_2	Saline	114.4 ± 5.3	138.8 ± 14.0	140.5 ± 9.1
(mm Hg)	L-NAME	121.1 ± 7.5	141.5 ± 5.0	136.7 ± 9.2
pH	Saline	7.40 ± 0.02	7.41 ± 0.01	7.40 ± 0.02
	L-NAME	7.39 ± 0.02	7.39 ± 0.01	7.38 ± 0.02
Hgb	Saline	13.6 ± 0.3	9.4 ± 0.2	6.7 ± 0.1
(gm/dL)	L-NAME	14.0 ± 0.2	9.4 ± 0.1	6.8 ± 0.1
MAP	Saline	98 ± 6	102 ± 4	101 ± 5
(mm Hg)	L-NAME	132 ± 5**	135 ± 3**	129 ± 5**

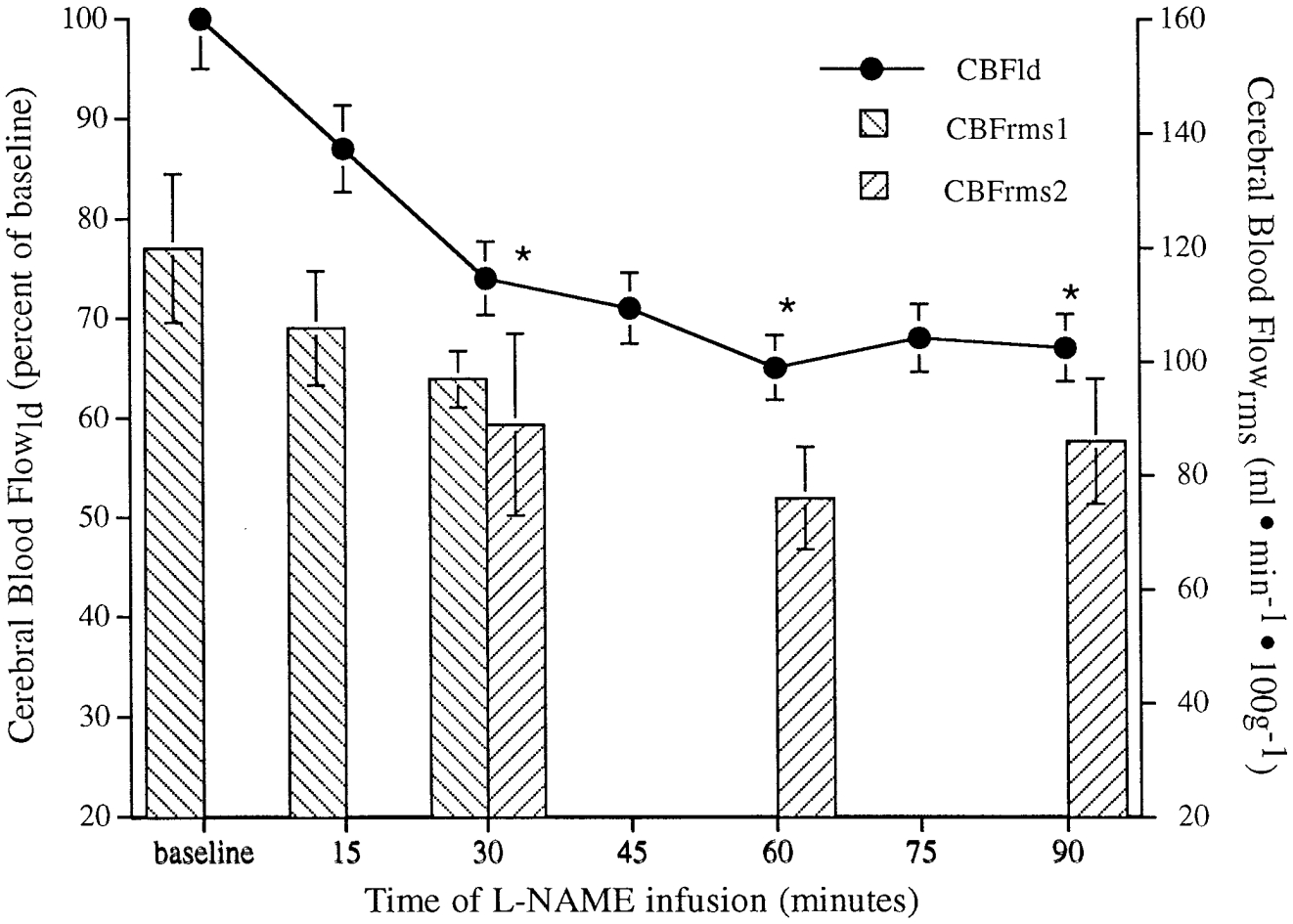
All values are mean ± SEM. ** significantly different from the saline treated group.

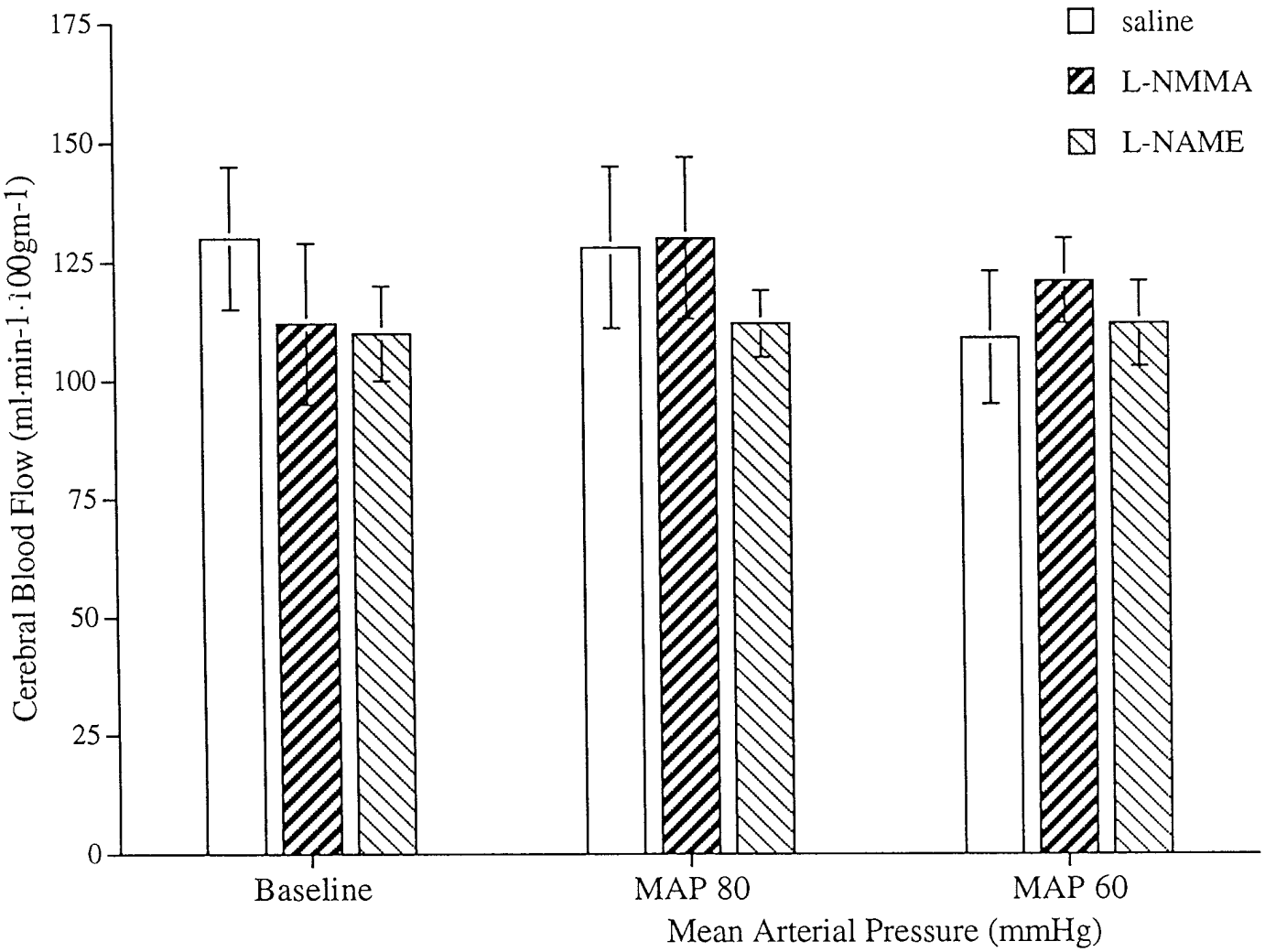
FIGURE LEGENDS

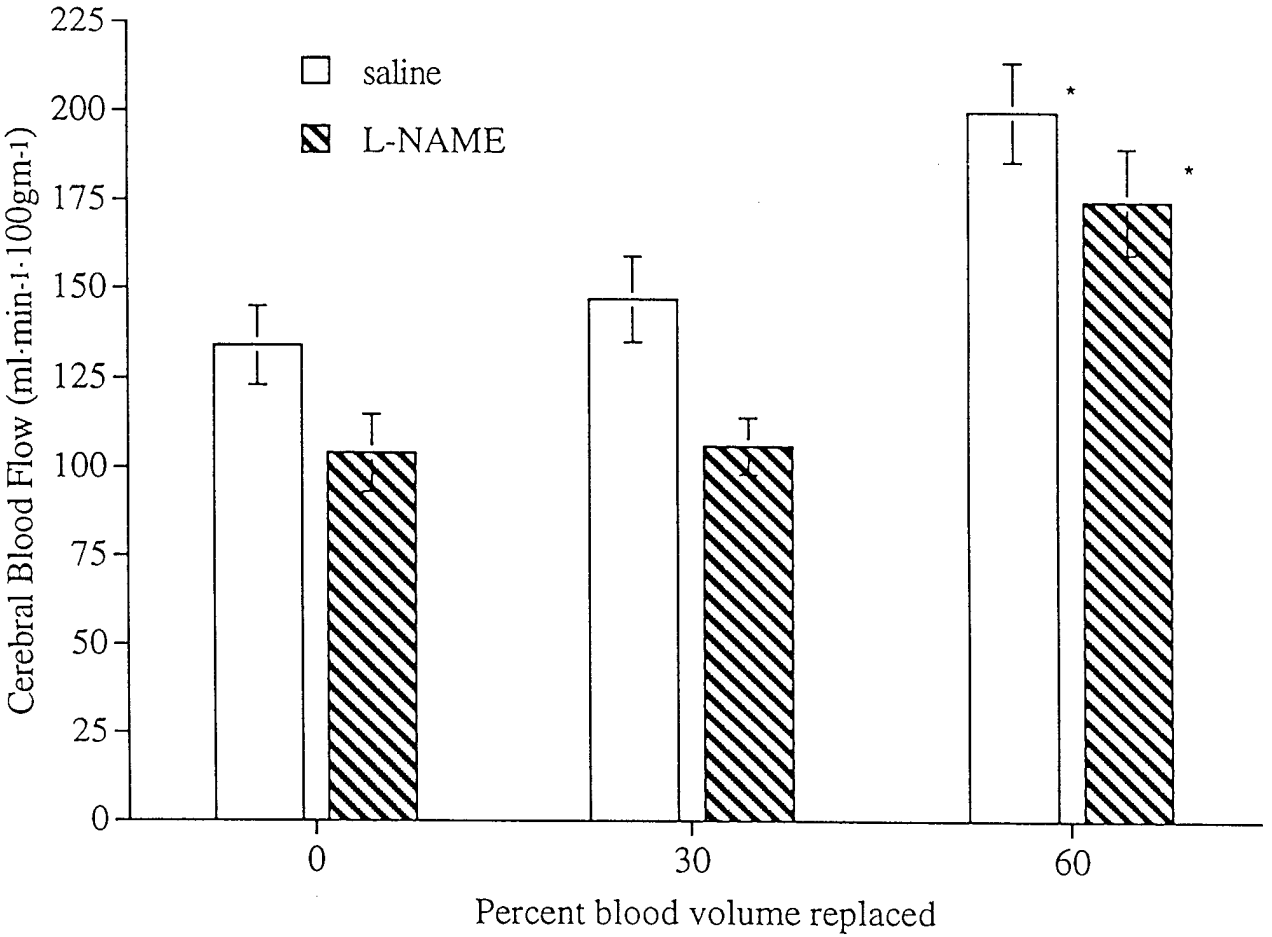
Figure 1. Cerebral blood flow (CBF) measured with radioactive microspheres (bars, right vertical axis) or laser Doppler flowmetry (LDF, solid line, left vertical axis) in rats treated with a continuous infusion of L-NAME (N^G-nitro-L-arginine methyl ester, 50 mg · kg^{-1} · min^{-1}). * significantly ($p < 0.05$) less than CBF at baseline. CBF_{ld} = CBF measured using laser Doppler flowmetry. CBF_{rms1} = CBF measured using radioactive microspheres before and 15 and 30 minutes after the start of L-NAME infusion. CBF_{rms2} = CBF measured using radioactive microspheres 30, 60, and 90 minutes after the start of L-NAME infusion.

Figure 2. Cerebral blood flow (CBF) at baseline (15 minutes after treatment) and after hemorrhage to mean arterial pressure (MAP) levels of 80 mm Hg and 60 mm Hg in rats treated with saline, L-NMMA (N^G-monomethyl-L-arginine, 25mg/kg) or L-NAME (N^G-nitro-L-arginine methyl ester, 25mg/kg).

Figure 3. Cerebral blood flow (CBF) at baseline (0–15 minutes after treatment with N^G-nitro-L-arginine methyl ester [L-NAME] , 25mg/kg, or saline) and after replacement of 30 and 60 percent of blood volume was exchanged for isotonic saline. a = significantly ($p < 0.05$) different from saline treated rats; * = significantly ($p < 0.05$) different from baseline (0).


CBFld
CBFrms1
CBFrms2
Cerebral Blood Flow1d (percent of baseline)
Cerebral Blood Flowrms (ml • min-1 • 100g-1)
100
90
80
70
60
50
40
30
20
160
140
120
100
80
60
40
20
*
*
*
baseline
15
30
45
60
75
90
Time of L-NAME infusion (minutes)


saline
L-NMMA
L-NAME
175
150
125
100
75
50
25
0
Cerebral Blood Flow (ml·min-1·100gm-1)
Baseline
MAP 80
MAP 60
Mean Arterial Pressure (mmHg)


225
200
175
150
125
100
75
50
25
0
saline
L-NAME
Cerebral Blood Flow (ml·min-1·100gm-1)
*
*
0
30
60
Percent blood volume replaced

CANNIBANOID RECEPTORS IN PERIVASCULAR SENSORY NERVES MEDIATE VASODILATION TO REDUCED INTRAVASCULAR PRESSURE IN RODENT CEREBRAL ARTERIES.

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The cerebral circulation is surrounded by a dense sensory nerve plexus which may contribute to cerebral vasodilatory responses to reduced intravascular pressure. Anandamide, an endogenous ligand of the cannabinoid 1 (CB1) receptor, is a potent cerebral vasodilator, but the role of anadamide or other CB1 receptor ligands in normal vasodilatory responses in the cerebral circulation is unknown. To determine whether CB1 receptors and perivascular sensory nerves are involved in cerebral vasodilatory responses to reduced intravascular pressure, the effects of the CB1 receptor anatagonist, SR141716A, the calcitonin gene-related peptide (CGRP) antagonist h-$CGRP_{8-37}$ or phenolic ethanol, which destroys perivascular nerves, on vasodilatory responses to progressive reductions in intravascular pressure were determined in rat middle cerebral arteries (MCA's). Rats were anesthetized with isoflurane, decapitated, their MCA's were harvested, mounted in an arteriograph and allowed to equilibrate. Intravascular pressure was then increased to 100 mmHg and changes in MCA inner diameters were measured as intravascular pressure was reduced progressively from 100 to 20 mmHg in 20 mmHg increments. Responses to reduced intravascular pressure were tested and then MCA's were exposed to SR141716A (3μM, intraluminally, n=6), h-$CGRP_{8-37}$ (1μM, n=6) or phenolic ethanol (0.75% phenol in 6.75% ethanol for 10 sec, n = 6) and responses were tested again. Before treatment, MCA's dilated to diameters greater than baseline at transmural pressures of 80, 60 and 40 mmHg and then decreased slightly as transmural pressure was reduced to 20 mmHg. After treatment with SR141716A or phenol, MCA diameters decreased sequentially as intravascular pressure was reduced progressively to 20 mmHg. After h-$CGRP_{8-37}$ exposure, MCA's dilated less than control MCA's in response to reduced intravascular pressure. Time control (n=5) and vehicle control (n=6) groups also were performed in which responses to reduced intravascular pressures were determined twice with 30 min or with ethanol treatment between tests. In both cases, vasodilatory responses were slightly reduced but still present during the second hypotensive challenge. These studies, which demonstrate that an inhibitor of CB1 receptors or phenol decrease vasodilatory responses to reduced intravascular pressure in MCA's, suggest that an agent in perivascular

sensory nerves acting a CB1 receptors may contribute to myogenic vasodilatory responses to reductions in intravascular pressure in MCA's *in vitro*.

Running Title: Cannabinoids and cerebral vascular reactivity

Keywords: cannabinoid, anandamide, SR141716A, cerebral arteries

INTRODUCTION

The cerebral circulation is surrounded by a dense sensory nerve plexus in the tunica adventitia of the cerebral blood vessels. The plexus is composed of nerve fibers that contain combinations of a dozen known or suspected neurotransmitters, including vasodilatory transmitters such as acetylcholine, substance P and calcitonin gene related peptide (CGRP){12628}. While the role of perivascular sensory nerves in normal cerebral vascular physiological responses is not known for certain, substance P and CGRP are believed to act to reduce excessive cerebral vasoconstriction and may contribute to cerebral vasodilatory responses to reduced blood pressure (autoregulation){12440, 12628}. Hong, et al., {12634} reported that pial arteriolar vasodilatory responses to systemic arterial hypotension were reduced by treatment with capsaicin (a depletor of perivascular neurotransmitters) or with antibodies to CGRP. Chronic trigeminal ganglionectomy, which decreased substance P levels and the density of CGRP-containing perivascular nerve fibers, reduced cortical hyperemic responses to ischemia by almost 60% (20). These observations suggest that perivascular sensory nerves contribute to normal cerebral vasodilatory responses and may help reduce brain injury due to ischemia or trauma.

In addition to substance P, CGRP and acetylcholine, recent evidence suggest that perivascular sensory nerves may contain or produce other important vasodilatory transmitters. Ishioka and Bukoski (14) reported that Ca^{2+}-induced relaxation in the mesenteric circulation may be due to the release of N-arachidonylethanolamine (anandamide) from perivascular sensory nerves. Ca^{2+}-induced relaxation, which results from hyperpolarization which is dependent on a Ca^{2+}-sensitive K^+ channel, can be blocked by SR141716A, a specific inhibitor of the cannabinoid 1 (CB1) receptor. CB1 receptors were originally described in the brain (6)(12) but functional CB1 receptors have subsequentially been discovered in spleen cells (15). CB2 receptors have been described in the spleen, thymus, tonsils and mast cells (24)(22). CB1 and CB2 receptors, which contain 472 and 360 amino acids, respectively, are G-protein coupled receptors with seven transmembrane domains (32)(1). Details of the structure, characterization and localization of

cannabinoid receptors are provided in several excellent reviews (26)(32)(1)(2).

CB1 messenger RNA is expressed peripheral vascular smooth muscle and endothelial cells (29)(5) and Δ^9-tetrahydrocannabinol and anandamide (arachidonyl ethanolamide), a putative endogenous ligand of the CB1 receptor, are potent cerebral vasodilators {11482}. These observations suggest the presence of CB1 receptors in the cerebral circulation but this has not, as yet, been demonstrated. While CB1 receptor ligands are capable of dilating the cerebral vascular, the role of these receptors in normal cerebral vasodilatory mechanisms is unknown. Therefore, we tested the hypothesis that CB1 receptors contribute to vasodilatory responses in reductions in intravascular pressure in isolated cerebral arteries and that the source of the endogenous ligands of the CB1 receptor are the perivascular sensory nerves.

METHODS

Using a protocol approved by the Instititutional Animal Care and Use Committee of The University of Texas Medical Branch, adult, male, Sprague Dawley rats (300-400 g) were anesthetized with 4% isoflurane, decapitated, the brains were removed and placed in cold physiological salt solution (PSS) and the MCA's were harvested. Middle cerebral arteries were mounted in an arteriograph (Living Systems, Burlington, VT) as described {12752}. Briefly, a section (2 mm) of the artery was mounted in the arteriograph by inserting micropipettes into the lumen at either end and securing the vessel with nylon suture (10-0). All side branches were tied off. The mounted arterial segments were bathed in PSS of the following composition (NaCl = 130 mM; KCl = 4.7 mM; $MgSO_4 \cdot 7H_2O$ = 1.17 mM; glucose = 5 mM; $CaCl_2$ = 1.50 mM, $NaHCO_3$ = 15 mM). When gassed with a mixture of 74% N_2, 21% O_2 and 5% CO_2, this solution has a pH of 7.4. After mounting, the PSS was warmed from room temperature to 37°C and the arterial segments were allowed to equilibrate for 60 min with intravascular pressure set at 50 mmHg by raising reservoir bottles connected to the micropipettes. Leaks were detected by monitoring intravascular pressure with the stopcocks to the reservoirs closed. A decline in pressure indicated a leak. After 1 hr of stabilization at an intravascular pressure of 50 mm Hg, each vessel was exercised to decrease mechanical hysteresis by changing the pressure between 20 and 40 mmHg 3 times, allowing 10 min between pressure changes. The vessels were magnified with an inverted microscope equipped with a video camera and a monitor. Arterial inner diameter was measured using a video scaler calibrated with an optical micrometer. Vasodilatory responses were tested by increasing or decreasing intravascular pressure in 20 mmHg increments with a 10-min equilibration period at each pressure level before diameter measurements are made. After the initial myogenic responses were evaluated, the MCA's were treated as described below. Vasodilatory responses were again tested by increasing or decreasing intravascular pressure in 20 mmHg increments. Following the second measurement of vasodilatory responses to progressive reductions in intravascular pressure, the Ca^{2+}-containing PSS was then replaced with Ca^{2+}-free

PSS and sequential responses to progressive reductions in intravascular pressure were assessed again.

For the experiments involving electrical stimulation, platinum electrodes were placed approximately 2 mm on either side of each MCA. The stimulus was a 0.6 ms square-wave pulse delivered at a current of 90 mA and a frequency that was varied between 0.4 and 2 Hz. MCA diameters were measured at the end of the stimulus. A stimulus sequence was performed, the MCA's were exposed to SR141716A, phenol, capsaicin or human calcitonin gene-related peptide$_{8-37}$ (h-CGRP$_{8-37}$) and a second stimulation sequence was performed.

Experimental Design

Myogenic Responses

Time Control - Two sequential meausurements of MCA diameter during progressive reductions in intravascular pressure were performed (n = 6).

Vehicle Control - Two sequential meausurements of MCA diameter during progressive reductions in intravascular pressure were performed. In between the two measurements, the MCA's were exposed to the vehicle for the SR141716A (ethanol,1.5%, intraluminally, n = 6).

PSS Control - In order to determine whether rapid removal and replacement of the PSS in the bath around the MCA's (necessary for the phenol experiments), two sequential meausurements of MCA diameter during progressive reductions in intravascular pressure were performed (n = 6). In between the two measurements, the PSS in the bathing chamber was suctioned out and immediately resplaced with fresh PSS (n = 6).

SR141716A - Two sequential measurements of MCA diameter during progressive reductions in intravascular pressure were performed. In between the two measurements, the MCA's were exposed to SR141716A (3 μM in ethanol, 1.5%, intraluminally, n = 6).

Phenolic ethanol - Two sequential measurements of MCA diameter during progressive reductions in intraluminal pressure were performed. In between the two measurements, phenolic ethanol (0.75% in 6.75% ethanol, n = 6) was added to the bath, allowed to remain for 10 sec and the

phenol-containing PSS was suctioned off and immediately replaced with fresh PSS.

h- $CGRP_{8-37}$ - Two sequential measurements of MCA diameter during progressive reductions in intravascular pressure were performed. In between the two measurements, the MCA's were exposed to h-$CGRP_{8-37}$ (1 μM, n = 6).

Electrical Stimulation

Time Control - Two sequential stimulation sequences were performed with no treatment between (n = 6).

SR141716A - Two sequential stimulation sequences were performed. In between, MCA's were exposed to SR141716A (3 μM in ethanol, 1.5%, intraluminally, n = 6).

Phenolic ethanol - Two sequential stimulation sequences were performed. In between the two measurements, phenolic ethanol (0.75% in 6.75% ethanol, n = 6) was added to the bath, allowed to remain for 10 sec and the phenol-containing PSS was suctioned off and immediately replaced with fresh PSS. Preliminary studies indicated that 10 s of exposure to phenol reduced myogenic responses to decreased intravascular pressure without affecting vasoconstrictory responses to serotonin.

Capsaicin - Two sequential stimulation sequences were performed. In between, MCA's were exposed to capsaicin added to the PSS in the bath (2 μM, n = 6).

h-$CGRP_{8-37}$ - Two sequential stimulation sequences were performed. In between, MCA's were exposed to $CGRP_{8-37}$ (1 μM, n = 6).

Statistical analysis

Data were normalized to a percent of baseline diameters and analyzed using analysis of variance for a two-factor experiment with repeated measures on time after exposure to SR141617A, phenolic ethanol, h-$CGRP_{8-37}$, etc. at each level of intravascular pressure or stimulus frequency. The other factor was treatment group (i.e. SR141617A, phenolic ethanol, h-$CGRP_{8-37}$, etc). Post-hoc analyses were performed using the Bonferroni/Dunn procedure for

multiple comparisons. All data in the text, table and figures are expressed as mean ± standard error of the mean.

RESULTS

Myogenic vasodilatory responses

In all groups before treatment and in the three control groups before and after treatment, MCA's dilated as intravascular pressure was reduced from 100 to 60 mmHg. MCA diameter decreased slightly when intravascular pressure was reduced to 40 and then 20 mmHg but, in most cases, MCA diameters remained above baseline (100 mmHg) at 20 mmHg. The exceptions were the pre-h-$CGRP_{8-37}$ group (Fig. 2C), exhibited vasodilation as intravascular pressure was reduced from 40 to 20 mmHg, and the pre-SR141716A group (Fig. 2A) which exhibited a slight reduction in diameter below baseline values at 20 mmHg of intravascular pressure.

There were no significant ($p = 0.11$) differences in the vasodilatory responses to progressive reductions in intravascular pressure between two successive measurements (Fig. 1A, Time 1 vs Time 2). This indicates that pre- and post-treatment measurements of vasodilatory responses can be used for these studies. There were no differences ($p = 0.08$) between the pre- (Fig. 1B, pre-EtOH) and post-treatment (Fig. 1B, post-EtOH) measurements in MCA's treated with the ethanol vehicle for SR141716A. Similarly, there were no significant differences in MCA diameters during reductions in intravascular pressure before (Fig. 1C, pre-PSS) and after (Fig. 1C, post-PSS) the PSS in the arteriograph bath was evacuated and quickly replaced with fresh PSS. These data indicate that the removal and replacement of PSS, which was required so the MCA's could be exposed to phenolic ethanol for only 10 sec, does not decrease vasodilatory responses to reduced intravascular pressure.

Intraluminal administration of SR141716A caused a modest ($12.3 \pm 9.0\%$) increase in baseline (100 mHg) diameter. MCA's treated with SR141716A exhibited vasoconstriction rather than vasodilation as intravascular pressure was successively reduced from 100 to 20 mmHg (Fig. 2A). There was a significant treatment effect ($p < 0.01$) observed in this group. Treatment with phenol also resulted in significant ($p < 0.01$) vasoconstrictor responses to reduced intravascular pressure (Fig. 2B). There was a significant ($p < 0.01$) treatment effect in the group treated with

$CGRP_{8-37}$, as well. There was neither vasodilation nor vasoconstriction as intravascular pressure was reduced to 80 and then 60 mmHg but vasoconstriction occurred as intravascular pressure decreased to 40 and 20 mmHg (Fig. 2C).

Responses to increasing frequency stimulation

There were significant treatment effects in the groups exposed to SR141716A ($p < 0.01$) or phenolic ethanol ($p < 0.05$). Increasing stimulation frequency caused vasodilation to some degree in all MCA's studied. SR141716A significantly decreased the amount of vasodilation observed in response to increased stimulation frequency (Fig. 3A). Exposure to phenol for 10 sec abolished vasodilation to 0.4 and 0.8 Hz stimulation but vasodilation occurred as stimulation frequency was increased to 1 and 2 Hz (Fig. 3B). Capsaicin reduced vasodilation to increases in stimulation frequency but there was no signficant treatment effect in the capsaicin group (Fig. 3C). h-$CGRP_{8-37}$ exposure did not affect vasodilatory responses to increasing stimulation frequency (Fig. 3C).

DISCUSSION

These studies demonstrated that the abolision of perivascular sensory nerves with a brief exposure to phenol *in vitro*, significantly reduces vasdilatory responses to electrical stimulation. The CB1 receptor anatagonist, SR141716A, also signficantly reduced vasodilation in MCA's during electrical stimulation. Our results further demostrated that destruction of perivascular nerves with phenol or that treatment with SR141716A reduced cerebral vasodilatory responses to progressive reductions in intravascular pressure *in vitro*. These data suggest that perivascular sensory nerves cotnribute to cerebral dilation in response to reduced intravascular pressure through an agent that acts at the CB1 receptor.

Our control studies demonstrated that vasodilatory responses to reduced intravascular pressure are reproducible across two sequential measurements (Fig. 1A). We further showed that neither the ethanol vehicle for SR141716A nor the evaculation and immediate replacement of the PSS bathing medium in the arteriograph significantly reduced vasodilation in response to decreased intravascular pressure (Fig. 1B & C). Therefore, the reductions in myogenic vasodilatory responses observed after exposure to SR141716A or phenol were due to these agents and not to the vehicle solutions or the method of administration.

SR141716A, which is 50 times more potent at CB1 (K_d: 12.3 nM) than at CB2 (K_d: 702 nM) receptors (27), is likely either a competetive antagonist or a reverse agonist of the CB1 receptor (32). SR141716A, at concentrations greater than 3 μM, produce endothelium-independent relaxations of rodent mesenteric arteries *in vitro* (35). At 10 μM, SR141716A causes relaxation by inhibiting Ca^{2+} entry and also inhibits relaxation mediated by the activation of K^+ channels and 5 μM SR141716A releases Ca^{2+} from intracellular stores (23). Thus, SR141716A at concentrations higher than those used in the present study, has non-specific effects not related to inhibition of CB1 receptors which limits its utility for studies of the effects of CB1 receptors on vascular function (35)(23). In the present study, 3 μM SR141716A produced modest (12.3 $\pm$ 9.0%), but statistically insignificant, relaxation of rodent MCA's.

Our observations that SR141716A decreases cerebral vasodilatory responses to reduced intravascular pressure or electrical stimulation in MCA's *in vitro* suggest that functional CB1 receptors are present on the cerebral arteries that are involved in these responses. CB1 receptors are present in the brain (6)(12) and other tissue (15). Evidence from in vivo studies suggest the presence of CB1 recetors on peripheral resistance vessels in rats but in vitro studies suggest that rat tail arteries have no CB1 receptors (21). CB2 cannabinoid receptors have been described in the spleen, thymus, tonsils and mast cells (24)(22). Δ^9-tetrahydrocannabinol and anandamide are potent cerebral vasodilators {11482} suggesting the presence of CB1 receptors in the cerebral circulation but this has not, as yet, been demonstrated. Cannabanoid receptors have been suggested to be the site of action of endothelium-derived hyperpolarizing factor(s) in the mesenteric circulation of rats (11), although this does not appear to be the case in others species such as rabbit (25). Recent evidence suggests the presence of an anandamide- and SR141716A-sensitive receptor in the rodent mesenteric circulation that is distinct from the known CB receptors (31). Our observations that the CB1 receptor anatagonist SR141716A reduces vasodilatory responses to decreases in intravascular pressure in cerebral arteries suggest that a CB1 ligand contributes to cerebral myogenic vasodilation but the nature of the endogenous CB1 ligand is not known.

To date, two endogenous ligands of the cannabanoid receptors have been described. Arachidonyl ethanolamine (anandamide) and 2-arachidonyl glyceride (2-AG) can be synthesized in the brain (8)(28) and both have been reported to affect brain activity (28)(4). Anandamide releases cytosolic Ca^{2+} from intracellular stores and increases NOS activity in cultured human umbilical vein endothelial cells (23). Anadamide is a potent dilator in the feline cerebral circulation {11482}. Anandamide has been implicated in the regualtion of systemic blood presuse and it may contribute to hypotenisoin during hemorrhagic shock (32)(19). Recent evidence indicates that vanilloid (capsaicin) receptors rather than cannabinoid receptors may mediate some fo the peripheral cardiovascular effects of anandamide but this remains to be tested in the cerebral circulation (36)(30).

The function of perivascular nerves in the cerebral circulation have been studied by denervating the cerebral vasculature surgically or pharmacologically (20). Recently, Wang & Bukoski (33) described a method to chemically denervate mesenteric arteries using a brief exposure to a phenolic ethanol solution (0.75% phenol in 6.75% ethanol). Using the same solution, we demonstrated that phenolic ethanol markedly reduced vasodilatory responses to electrical stimulation in MCA's *in vitro* (Fig. 3B). Phenol also significantly reduced vasodilatory responses to progressive decreases in intravascular pressure in MCA's *in vitro* (Fig. 2B). In contrast to the inhibitory effects of phenolic ethanol on vasodilatory responses to electrical stimulation or reduced intravascular pressure, brief exposure to phenol did not reduce vasoconstrictor responses to serotonin. After exposure to phenol, 10^{-6} M serotonin reduced MCA inner diameter to 64.3 ± 2.3% of baseline compared to reductions in diameters to 63.4 ± 5.8% of baseline in control MCA's from a previous study (7). These results suggest that phenol reduces vasodilatory responses mediated by perivascular sensory nerves but does not impair the ability of the arterial smooth muscle to constrict normally.

Human $CGRP_{8-37}$ is a competative antagonist of $CGRP_1$ receptors (3) which inhibits the vasodilatory effects of electrical stimulation of canine lingual arterial rings *in vitro* (17). Increases in meningeal artery blood flow induced by electrical stimulation in rats are inhibited in a dose-dependent manner by h-$CGRP_{8-37}$ (18). In contrast, we observed no effect of h-$CGRP_{8-37}$ on MCA vasodilation in response to electrical stimulation (Fig. 3D). It is not clear why we observed no effect of h-$CGRP_{8-37}$ on vasodilatory effects of electrical stimulation on MCA's but evidence from the porcine coronary circulation indicates that large coronary arteries are 10-fold less sensitive to h-$CGRP_{8-37}$ are small coronary arteries (10). Perhaps h-$CGRP_{8-37}$ is less effective in large cerebral arteries such as the MCA. h-$CGRP_{8-37}$ reduced but did not abolish vasodilatory responses to decreases in intravascular pressure *in vitro* (Fig. 2C). These observations are consistent with the hypothesis that h-$CGRP_{8-37}$ does not completely inhibit the $CGRP_1$ receptor in

large arteries.

Previous evidence that antibodies to CGRP reduced cerebral arteriolar responses to systemic arterial hypotension *in vivo*, suggest that CGRP-containing perivascular nerves contribute to autoregulatory cerebral vasodilatory responses to systemic arterial hypotension, *in vivo* {12634} but our results suggest that, in rodent MCA's, CGRP-containing perivascular nerves play a relatively minor role. This is supported by our observations that capsaicin produced small, statsitically insignificant reductions in stimulation-induced vasodilation (Fig. 3C). Capsaicin releases and depletes neurotransmitters such as substance P and CGRP from sensory nerves (34)(9). This evidence that the depletion of CGRP had only a modest effect on vasodilatory responses to electrical stimulation when compared to the effects of SR141716A (Fig. 3A) suggest that CB1 receptor ligands may play a more important role in stimulation-induced vasodilation than does CGRP. An endogenous CB1 ligand such as anandamide would not be affected by capsaicin since anandamide does not appear to be stored in significant quantatives (16) but, rather, is synthesized as needed (13)(32).

In summary, our results are consistent with the hypothesis that CB1 receptors in cerebral vascular smooth muscle mediate vasodilatory responses to reduced intravascular pressure or electrical stimulation and that perivascular nerve fibers release vasodilatory neurotransmitters such as anandamide and, to a lesser extent, CGRP in response to changes in the intravascular pressure in the arteries they surround.

ACKNOWLEDGMENTS

The authors thank Jordan Kicklighter for valuable editorial and secretarial support. We are grateful to our colleagues in the Department of Anesthesiology at the University of Texas Medical Branch for their support of the Charles R. Allen Research Laboratories. These studies were supported by grant number DAMD17-97-1-7008 from the Department of the Army and grant numbers NS19355 and NS27618 from the NIH.

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Table 1. Middle cerebral artery inner diameters (μM) during progressive reductions in intravascular pressure in control groups. Group "time" involved two subsequent measurements of myogenic responses to reductions in intravascular pressure with no treatment in between. Group "EtOH" involved meausurement before (pre) and after (post) the ethanol vehicle of SR141716A was added to the bath. Group "PSS" involved measurements before and after the PSS vehicle of the phenol was added to the bath.

Group	Time	Intravascular Pressure (mmHg)				
		100	80	60	40	20
Time	pre	131±10	146±13	153±9	151±8	139±8
	post	114±9	124±13	128±13	125±13	114±7
EtOH	pre	159±4	169±5	173±8	165±14	160±13
	post	159±7	166±8	164±10	158±12	147±14
PSS	pre	160±19	165±20	171±20	170±20	166±20
	post	152±19	157±20	162±18	157±20	157±22

Table 2. Middle cerebral artery inner diameters (μM) during progressive reductions in intravascular pressure before (pre) and after (post) exposure to SR141716A (3 μM), phenolic ethanol (0.75% phenol in 6.75% ethanol) or $CGRP_{8-37}$ (5 μM).

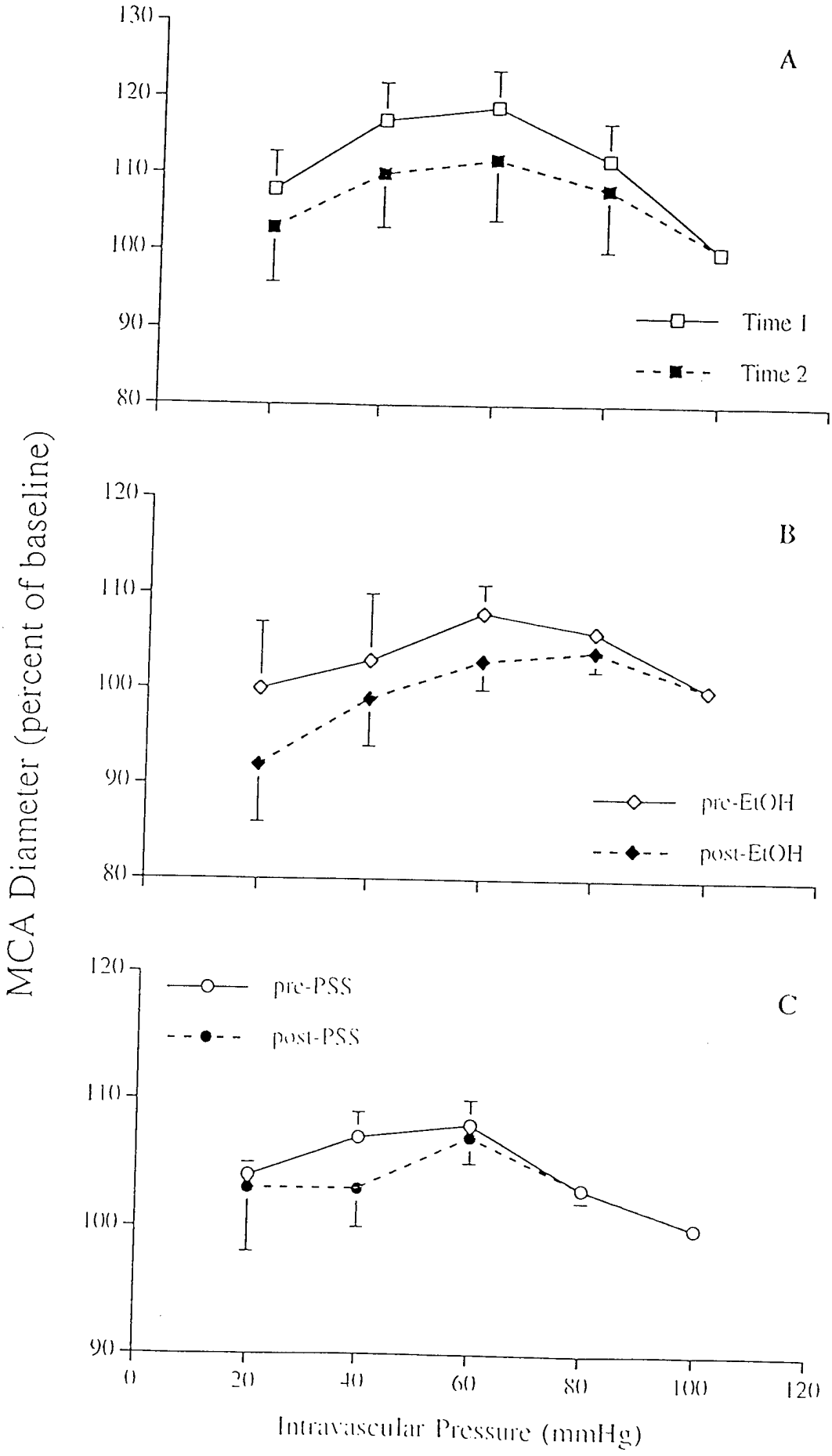
Group	Time	Intravascular Pressure (mmHg)				
		100	80	60	40	20
SR141716A	pre	132±12	142±12	146±12	140±11	127±12±6
	post	152±24	137±21	129±19	121±19	104±17
Phenol	pre	147±8	153±7	166±8	162±8	156±8
	post	123±9	112±5	106±5	102±5	98±6
$CGRP_{8-37}$	pre	107±6	110±5	120±8	122±8	114±8
	post	110±8	108±8	108±8	105±6	99±4

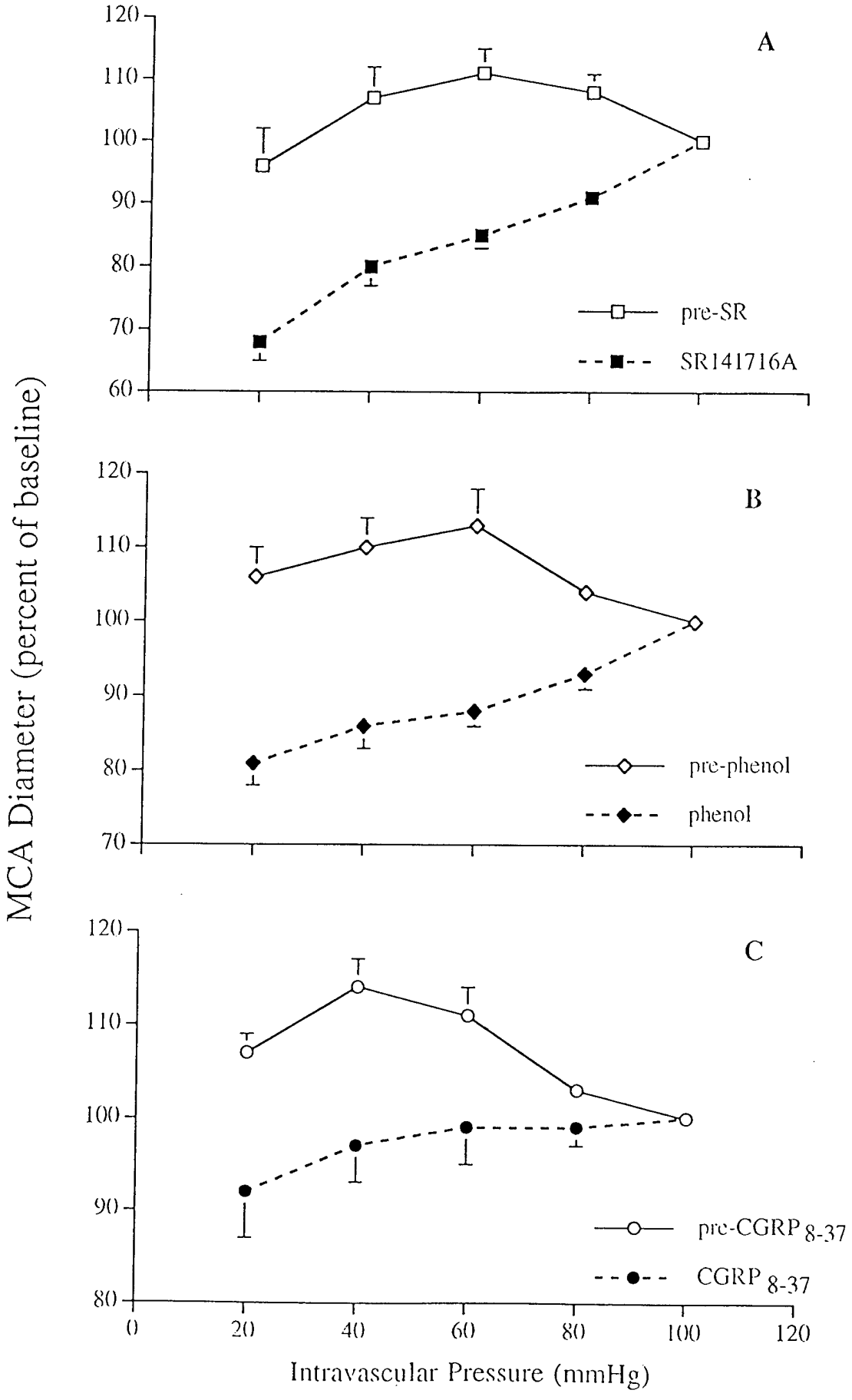
FIGURE LEGENDS

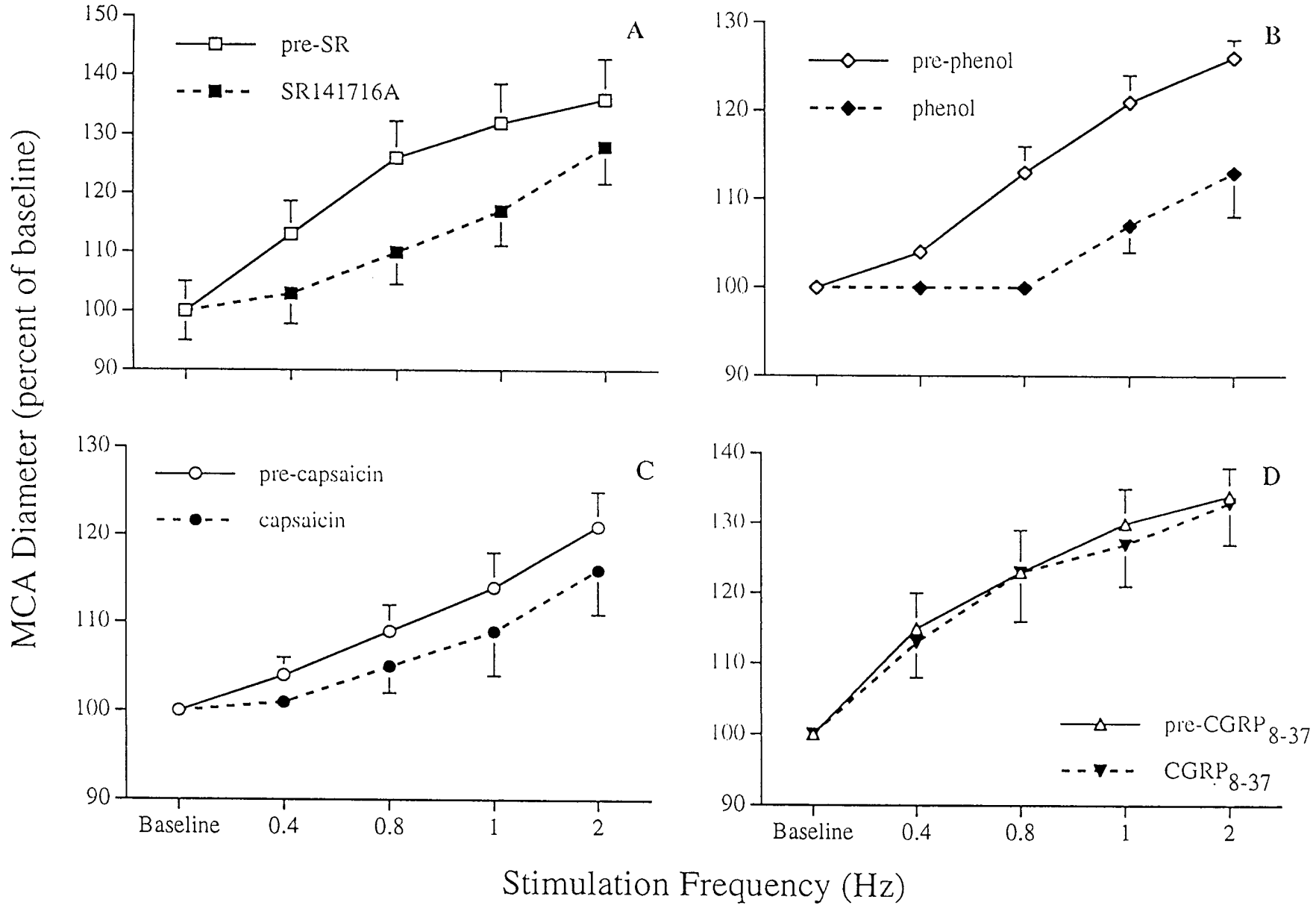
FIGURE 1 Middle cerebral artery (MCA) diameters (percent of diameter at 100 mmHg) during progressive reductions in intravascular pressure in control groups. Figure 1A is two successive measurements (Time 1 and Time 2) of MCA diameters during reductions in intravascular pressure. Figure 1B is pre- and post-treatment with the ethanol vehicle of SR1417165A. Figure 1C is pre- and post-removal and replacement of physiological salt solution (PSS), the control group for the phenol studies.

FIGURE 2 Middle cerebral artery (MCA) diameters (percent of diameter at 100 mmHg) during progressive reductions in intravascular pressure before and after treatment with SR141716A (2A), phenol (2B) or $CGRP_{8-37}$ (2C).

FIGURE 3 Middle cerebral artery (MCA) diameters (percent of pre-stimulation baseline) during increasing stimulation frequency before and after treatment with SR141716A (3A), phenol (3B), capsaicin (3C) or CGRP $CGRP_{8-37}$ (3D).


A
130
120
110
100
90
80
Time 1
Time 2
B
120
110
100
90
80
pre-EtOH
post-EtOH
C
120
110
100
90
pre-PSS
post-PSS
0
20
40
60
80
100
120
MCA Diameter (percent of baseline)
Intravascular Pressure (mmHg)


A
B
C
MCA Diameter (percent of baseline)
Intravascular Pressure (mmHg)
pre-SR
SR141716A
pre-phenol
phenol
pre-CGRP 8-37
CGRP 8-37
120
110
100
90
80
70
60
0
20
40
60
80
100
120


MCA Diameter (percent of baseline)
A
pre-SR
SR141716A
B
pre-phenol
phenol
C
pre-capsaicin
capsaicin
D
pre-CGRP8-37
CGRP8-37
Baseline
0.4
0.8
1
2
Stimulation Frequency (Hz)